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TESIS DOCTORAL

Utilidad médica de la identificación de los hongos patógenos mediante métodos moleculares

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Aunque para muchos este estas son las primeras hojas de una tesis más, para mi es el final de una etapa. Una etapa en la que no he parado de aprender y en la que he disfrutado de tantas cosas y tanta gente que ahora me resulta muy difícil poder expresar a todos mi gratitud. Con estas líneas me gustaría poder enumerar y agradecer a todas estas personas que de alguna manera han contribuido a que se realice este trabajo y a que yo haya podido llegar hasta aquí.

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Resumen

La incidencia de las infecciones fúngicas ha seguido aumentando en los últimos años por lo que se han convertido en una de las principales causas de morbilidad y mortalidad en pacientes inmunocomprometidos. Los agentes etiológicos más frecuentes siguen siendo *Candida albicans* y *Aspergillus fumigatus*, sin embargo, el número de especies fúngicas capaces de causar infecciones invasoras ha aumentado considerablemente. Estas infecciones son más difíciles de diagnosticar y tratar ya que estas especies emergentes son más resistentes a los antifúngicos y por tanto están asociadas con mayores tasas de mortalidad.

Este cambio en la epidemiología unido al aumento en el número de antifúngicos disponibles para tratar las infecciones y las diferencias de sensibilidad que presentan las distintas especies, subrayan la importancia de una correcta identificación a nivel de especie. Las técnicas clásicas para la identificación de hongos filamentosos son lentas, difíciles y exigen de un gran conocimiento de la taxonomía, ya que se basan en la observación de las características morfológicas. Recientemente un grupo de expertos ha designado a las regiones ITSs del rDNA la técnica de elección para la identificación de los hongos patógenos humanos. No hay que olvidar, que otra herramienta muy útil en el manejo de las infecciones causadas por hongos es el estudio de su perfil de sensibilidad a los antifúngicos disponibles.

El principal objetivo de este trabajo fue desarrollar herramientas de identificación de los principales hongos patógenos humanos y correlacionar esta identificación con el perfil de sensibilidad a los antifúngicos disponibles para tratar dichas infecciones.

De los resultados obtenidos se puede concluir: (1) La secuenciación de la región ITS constituye un método preciso para la identificación de los mucorales a nivel de especie y la clasificación de cepas de *Aspergillus, Fusarium* y *Scedosporium* dentro de la sección a la que pertenecen, pero especialmente para la identificación de una cepa *a priori* desconocida. La secuenciación de genes codificantes aporta la suficiente variabilidad para llegar a nivel de especie en los géneros *Aspergillus, Fusarium* y *Scedosporium.* En el caso de *Aspergillus y Scedosporium* la identificación a nivel de especie se consigue mediante la secuenciación de la β tubulina, mientras que en el genero *Fusarium* la diana más apropiada es una región del factor de elongacion 1 α. (2) Con respecto a los perfiles de sensibilidad a los antifúngicos, los resultados derivados de la comparación de los estudios de sensibilidad dentro de cada grupo de hongos estudiados permitió concluir que la identificación a nivel de especie en *Aspergillus* sección *Fumigati* permite la clasificación de las especies de *Aspergillus* de esta sección dentro de cada uno de los perfiles de sensibilidad existentes. Por el contrario, las especies de *Aspergillus* sección *Usti* no presentan diferencias significativas en sus perfiles de sensibilidad, por lo que la identificación a nivel de complejo de especies dentro de esta sección podría ser suficiente para dar el tratamiento más correcto. El perfil de sensibilidad de *Aspergillus* sección *Nigri, Fusarium* spp. y mucorales es dependiente de cepa. Por tanto, se recomienda realizar los test de sensibilidad a las cepas implicadas en los infecciones probadas y probables. (3) Con los datos de esta tesis se ha desarrollado una base de datos de secuencias de ADN y sensibilidad a los antifúngicos que representa una herramienta robusta y fiable. Esta base de datos tiene una aplicabilidad directa en las funciones que desempeña el Servicio de Micología del Centro Nacional de Microbiología, ya que se utiliza para la identificación molecular mediante comparación de secuencias. Además en base a ella se han desarrollado sondas específicas de los principales hongos patógenos humanos para el desarrollo de técnicas de PCR a tiempo real y microarrays de identificación con el fin de facilitar el diagnóstico de las infecciones fúngicas.

Summary

Fungal infections have significantly increased in frequency and as causes of morbidity and mortality, especially among inmunocompromised hosts. *Candida albicans* and *Aspergillus fumigatus* remain the most frequent etiologic agents of these infections, however, novel organisms not previously associated with human infections are being reported as causative agents of infection. Infections caused by these emerging fungal pathogens are more difficult to diagnose and treat since they are frequently more resistant to available antifungal drugs. Consequently, they are associated with higher mortality rates.

The changing epidemiology, the higher number of antifungal agents available and the differences in susceptibility profiles, highlight the need of a proper identification to species level of the etiologic agent of the infection. Classically, the identification of fungi has been based in the recognition of morphological characteristics and therefore is a cumbersome and time consuming task. Few microbiologists are able to properly identify human pathogenic fungi because it requires a high level of classical mycological training. A group of experts have recently designated the ITSs regions of the rDNA as the standard locus for species identification. In addition to the species identification, the determination of the susceptibility profile is another useful tool that allows the proper management of fungal infections.

The main objective of this thesis was to develop molecular biology tools that allow the identificacion of the main mould fungal pathogens, and to correlate this identification with the antifungal susceptibility profile to the main antifungals.

From the results obtained, it can be concluded: 1) The ITS sequencing is a reliable method for the accurate identification of most medically important mucorales to species level and for identification of *Aspergillus, Fusarium* and *Scedosporium* isolates to the species complex level. But this technique has been especially useful for the identification of unknown strains. Protein coding genes should be used in *Aspergillus, Fusarium* and *Scedosporium* to identify species level. Thus □-tubulin has enough variability to reach species level in *Aspergillus* spp. and *Scedosporium* spp., while in the *Fusarium* genus, the elongation factor 1-□ is the targeted locus for this purpose. (2) Concerning susceptibility profiles, in *Aspergillus* section *Fumigati* the identification to species level allows the classification of these species into one of the susceptibility profiles present. However, in *Aspergillus* section *Usti,* there are no significant differences, thus the identification to section level could be enough to suggest the proper treatment. The susceptibility profile in *Aspergillus* section *Nigri, Fusarium* spp. and mucorales is strain-dependent. For this reason, the determination of the susceptibility profile in these species is mandatory in any probable or proven infection. (3) The work presented in this thesis has allowed the creation of a DNA and susceptibility profile database of the main human fungal pathogens. It represents a robust and trustable tool that nowadays is used in the Mycology Reference Laboratory of the Spanish National Centre for Microbiology to identify the isolates from the hospitals by means of molecular biology methods. This database has also been used to design specific probes for the main human fungal pathogens to develop real time PCR protocols and a microarray platform which can be used for the diagnosis of fungal infections.

Índice

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1. Introducción

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1.1. Importancia infecciones fúngicas

La incidencia de las infecciones fúngicas ha seguido aumentando en los últimos años por lo que se han convertido en una de las principales causas de morbilidad y mortalidad en pacientes inmunocomprometidos. Así, desde 1980 los Institutos de Salud Americanos cifran en un 10% el aumento anual de las infecciones fúngicas[127]. Otros estudios, también realizados en EEUU entre 1979-2000, detectan un incremento del 207% en las sepsis causadas por hongos[77] ocupando en 1992 el séptimo lugar como causa de muerte debido a una enfermedad infecciosa. Todos estos datos indican un incremento superior al 300% desde 1980[108]. En Europa, aunque los datos epidemiológicos a este respecto son muy escasos, el análisis de 8.124 necropsias de un hospital en Alemania, mostró un aumento del 2,9% en muertes causadas por hongos entre principios de la década de los ochenta y principios de la década de los noventa[47].

El aumento de la incidencia de la infección fúngica, está relacionado con la existencia de una mayor población de pacientes con factores predisponentes. Entre ellos cabe destacar el trasplante de progenitores hematopoyéticos y de órgano sólido, los nuevos quimioterápicos y la utilización de corticoides a altas dosis. Además, hay otros factores de riesgo que contribuyen a este aumento de incidencia como son el ingreso en unidades de cuidados intensivos y algunas intervenciones quirúrgicas[117]. Por último, a este aumento de la población de riesgo también contribuye el desarrollo científico y tecnológico de la medicina que ofrece alternativas a pacientes con edades avanzadas y con procesos que antes carecían de tratamiento. Además, la mortalidad asociada a las infecciones fúngicas es muy elevada, aunque está condicionada por el tipo de enfermedad y el perfil del huésped. En general, para la candidemia es alrededor del 40%, y para la aspergilosis invasora del 85%[28,95].

Actualmente, se han descrito 72.000 especies de hongos, frente a las 4.000 especies aceptadas de bacterias y virus. No obstante, se sospecha que existen al menos 1.500.000 especies fúngicas[54]. Anualmente se catalogan 1.700 hongos nuevos en el *Index of Fungi*, de los cuales al menos 20 son patógenos humanos. Esto significa, que en una década se describen 200 nuevos hongos capaces de causar infecciones en humanos. Por lo tanto, aunque los agentes etiológicos más frecuentes siguen siendo *Candida albicans* y *Aspergillus fumigatus*, el número de especies capaces de causar infecciones invasoras ha aumentado considerablemente. Así, en los últimos años han entrando en escena otras especies de *Candida* (no *albicans*), de *Aspergillus* (no

fumigatus), y otros géneros como *Rhodotorula, Trichosporon, Fusarium, Scedosporium* y mucorales. Las infecciones debidas a estos hongos son más difíciles de diagnosticar y están asociadas con una mayor tasa de mortalidad ya que en general son más resistentes a los antifúngicos[126].

1.2. Importancia de la identificación a nivel de especie

La identificación de los hongos a nivel de especie se ha hecho necesaria por múltiples razones: (i) el aumento del número de especies capaces de causar infecciones, (ii) la ampliación de las opciones terapéuticas debido a la aparición de nuevos antifúngicos y (iii) los diferentes perfiles de sensibilidad a los mismos. Así, *Trichosporon asahii* es resistente a la anfotericina B[118], que es el tratamiento de elección para las infecciones causadas por mucorales que son resistentes al voriconazol, siendo este último, en cambio, el fármaco de elección para la aspergilosis[4]. Estas diferencias también se encuentran en especies del mismo género, por ejemplo voriconazol y ravuconazol son activos frente a *Paecilomyces lilacinus* pero tienen baja actividad frente a *Paecilomyces variotii* que por el contrario es sensible a la anfotericina B, itraconazol y equinocandinas[17].

1.2.1. Identificación morfológica frente a identificación molecular

Clásicamente, la identificación al nivel de especie de los hongos se ha realizado mediante el estudio de sus características morfológicas y fenotípicas (pruebas bioquímicas, crecimiento a diversas temperaturas, etc.). Sin embargo, mientras que la identificación de una bacteria se hace en menos de 24 horas, la identificación de un hongo, puede demorarse días, semanas o meses. La identificación de las levaduras es similar a la que se utiliza con las bacterias empleando kits comerciales basados en la asimilación de diversas fuentes de carbono y nitrógeno. Por el contrario, la identificación de los hongos filamentosos se basa en la observación microscópica de los aislamientos, examinando estructuras como el tipo de talo, su morfología, la forma de las células conidiógenas y las conidias, así como las estructuras de reproducción sexual[27]. Todo ello, exige una gran formación y experiencia en el campo, además de la utilización de medios de cultivo especiales y tiempos prolongados de incubación. Aun así, en aquellas ocasiones en las que no se consigue la esporulación en cultivo, la identificación morfológica no es posible. Todo esto ha llevado a la búsqueda de alternativas más rápidas, fáciles y fiables. Entre todas ellas, la secuenciación de fragmentos informativos ha sido elegida como técnica de referencia ya que es objetiva y los datos obtenidos por cualquier laboratorio del mundo pueden ser intercambiados y utilizados por otros grupos. Su uso ha puesto al descubierto que existen especies crípticas dentro de grupos morfológicamente homogéneos que además tienen diferente perfil de sensibilidad a los antifúngicos.

El éxito de una correcta identificación por métodos moleculares mediante comparación de secuencias, se basa principalmente en la elección del marcador adecuado que debe tener las siguientes características: (i) que sea lo suficientemente variable para diferenciar entre las especies del género, pero lo suficientemente conservado para que las secuencias se puedan alinear con alguno de los programas disponibles; (ii) que se pueda amplificar con iniciadores universales (iii) que tenga un tamaño que permita una fácil amplificación y secuenciación.

El gen que codifica la citocromo C oxidasa es universalmente aceptado como gen de referencia para la identificación de las especies del reino animal. Sin embargo, en los hongos este gen evoluciona muy lentamente no permitiendo la distinción entre especies, por lo que se han buscado otros marcadores. Así, la región que codifica el RNA ribosómico (rRNA), es una familia de genes multicopia dispuestos en tándem. Cada copia tiene regiones codificantes 18S, 5,8S y 26S separadas por regiones no codificantes denominadas ITS (Internal transcribed spacer). Entre cada copia se encuentran las regiones IGS1 e IGS2 (Inter genic spacer) separadas por el gen que codifica el rRNA 5S (Figura 1). De todas ellas, la región ITS es la que cumple un mayor número de requisitos para ser utilizada universalmente en la identificación de hongos: (i) consta de dos regiones, ITS1 e ITS2, suficientemente variables para identificar a nivel de especie, separadas por el gen que codifica el rRNA 5,8S, región conservada que a su vez facilita el alineamiento de las secuencias, (ii) existen parejas de cebadores universales denominados ITS1 e ITS4 (Figura 1) que amplifican dicho fragmento[144], (iii) el tamaño del fragmento amplificado con estos iniciadores, tiene normalmente entre 400 y 800 pares de bases lo que permite ser amplificado y secuenciado de forma fácil y rápida. Además, la repetición en tandem de esta zona facilita su amplificación cuando hay poca cantidad de ADN. Por estas razones, recientemente un grupo de expertos ha acordado que la secuenciación de esta región del ADN nuclear (ITS1-5,8S-ITS2) sea la técnica de referencia para la identificación de las especies de hongos patógenos humanos[7].

Para conseguir una correcta identificación molecular, se necesita además una base de datos de secuencias que debería cumplir los siguientes requisitos: (i) que la identificación a través de la comparación de la secuencia se realice de forma sencilla e inequívoca mediante herramientas universalmente aceptadas, (ii) que cubra lo más ampliamente posible la variabilidad taxonómica del grupo con el que se compara la secuencia problema, (iii) y por último que las secuencias estén correctamente identificadas y anotadas. Actualmente, las bases de datos más utilizadas para la identificación molecular mediante comparación de secuencias son GenBank del NCBI (National Center for Biotechnology Information en EE.UU. http://www.ncbi.nlm.nih.gov/Genbank/index.html),

EMBL-Bank (European Molecular Biology Laboratory http://www.ebi.ac.uk/embl/) y DDBJ (DNA Data Bank of Japan http://www.ddbj.nig.ac.jp/index-e.html). Estas bases de datos son públicas, de acceso libre y están conectadas por lo que comparten la información depositada. En el caso de los hongos, las secuencias disponibles representan escasamente el 1% de las especies existentes y además, se ha estimado que cerca de un 20% están mal identificadas[15,88]. Existe un problema añadido, ya que muchas especies tienen dos nombres, uno indicativo de su estado asexual (anamorfo) y otro de su estado sexual (teleomorfo) que es el que aparece preferentemente en las bases de datos mencionadas. Sin embargo, en la micología médica este hecho genera bastante confusión, ya que el teleomorfo raramente se produce en cultivo o lo hace tan lentamente que no es útil desde el punto de vista práctico y habitualmente se denomina a los hongos por su anamorfo. Asimismo, la aplicación de las herramientas moleculares está dejando obsoletos numerosos nombres de especies pero estos cambios taxonómicos no siempre son actualizados en las bases de datos. Por tanto, para poder llegar a una correcta identificación hay que desarrollar bases de datos depuradas, robustas y fiables que contengan secuencias tipo o cepas de referencia de los organismos a analizar.

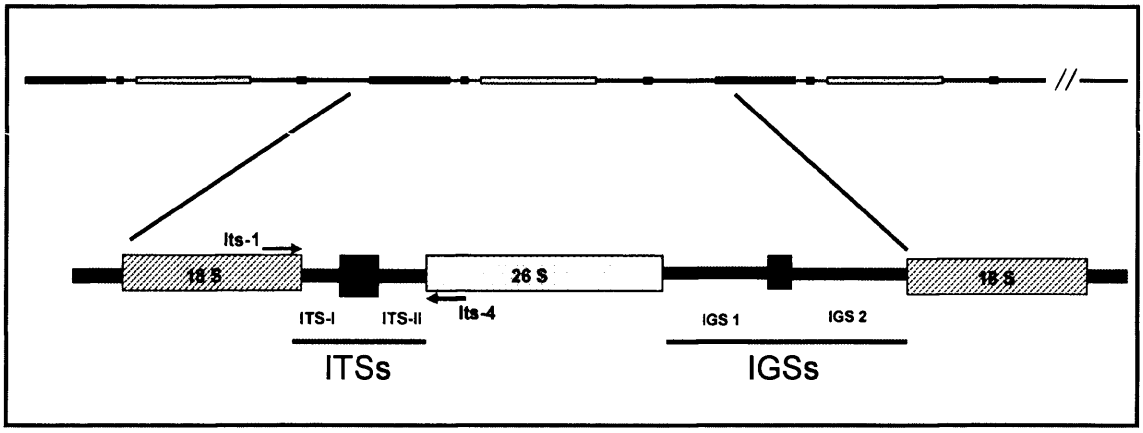


Figura 1. Representación esquemática del DNA que codifica el RNA ribosomal.

1.3. Principales grupos de hongos filamentosos patógenos humanos

Como se ha señalado anteriormente, la identificación de los hongos filamentosos presenta mayores problemas que la de las levaduras debido a que está basada en la experiencia del observador. Además, estos hongos están asociados con mayores tasas de mortalidad. Así, en 1992 el análisis de las necropsias de un hospital terciario concluyó que la causa de muerte del 7% de los enfermos había sido una infección fúngica causada en el 70% por hongos filamentosos y en el 30% por *Candida* spp.[31]. Por estas razones

este grupo de hongos es el que se ha sometido a estudio. A continuación describen los principales grupos de hongos filamentosos patógenos humanos.

1.3.1. *Aspergillus*

Aspergillus es un hongo ubicuo que puede causar desde alergias a infecciones diseminadas en pacientes inmunodeprimidos. El género incluye unas 175 especies, de las que más de 20 causan infecciones en humanos[48,75,76,78,84,97,102,114,137,141], con una tasa de mortalidad asociada del 80 al 90%[29,73,101,112,141]. Los teleomorfos de *Aspergillus* se encuentran distribuidos en varios géneros: *Neosartorya, Petromyces, Eurotium, Emericella, Fennelia, etc.*

A. fumigatus es la especie más frecuentemente asociada a las aspergilosis invasora. Durante los años 90 fue responsable del 90% de los casos de infecciones por hongos filamentosos[73,76]. Sin embargo, un estudio multicéntrico realizado entre los años 2001 y 2006 que analizó la infección fúngica invasora causada por hongos filamentosos en pacientes trasplantados de progenitores hematopoyéticos u órgano sólido ha demostrado un cambio en la epidemiología de las infecciones por *Aspergillus.* Así sólo un 67,4% de los aislados eran *A. fumigatus,* mientras que el 29% correspondieron a *Aspergillus flavus*, el 8,7 % *Aspergillus niger* y el 7,4% *Aspergillus terreus*[9]. El incremento en el número de infecciones causadas por *A. terreus* es de especial importancia, debido a su menor sensibilidad a la anfotericina B[133,143] y a su potencial para causar infecciones pulmonares invasivas en pacientes inmunodeprimidos. Badley et al.[6] encontraron un aumento del 2,1% en 1996 a 10,2% en 2001 y Balajee et al.[9] confirman una incidencia del 7,4% en pacientes trasplantados entre 2001 y 2006. Además, mediante la aplicación de técnicas moleculares se han descrito especies crípticas en varias secciones de este género[59,121], siendo algunas de ellas como *Aspergillus ustus* o *Aspergillus lentulus* más resistentes a los antifúngicos disponibles[8,98]. Recientemente, Balajee et al.[9] han identificado mediante herramientas moleculares 218 aislados procedentes de pacientes con aspergilosis invasora, encontrando que más de un 10% de las cepas eran especies crípticas de *Aspergillus,* lo que pone de manifiesto la importancia de la correcta identificación de las especies de este género.

1.3.2. *Fusarium*

Las especies del género *Fusarium* son frecuentes patógenos de plantas, sin embargo, en los últimos años ha aumentado mucho el número de infecciones causadas por especies de este género en humanos. Estas infecciones presentan unas tasas de mortalidad muy elevadas[19,89], llegando a superar el 75%[90]. *Fusarium solani* y *Fusarium oxysporum* son las

especies más preponderantes aunque las infecciones causadas por otras especies son cada vez más frecuentes[41,51,53]. Su identificación a nivel de especie se basa en las características microscópicas de las macroconidias, pero las cepas aisladas de muestras clínicas no suelen producir dichas estructuras, por lo que su identificación es muy difícil[14,27]. Las especies del género *Fusarium* son resistentes in vitro a la mayoría de los antifúngicos disponibles. Algunos datos apuntan que esta resistencia puede ser dependiente de especie o incluso de la cepa[22].

1.3.3. Mucorales

Los hongos del orden mucorales causan infecciones graves, de rápida evolución y frecuentemente fulminantes en pacientes inmunodeprimidos. Las especies más frecuentes pertenecen al género *Rhizopus*, aunque otras especies de los géneros: *Mucor, Rhizomucor, Lichtheimia* (syn. *Mycocladus*, *Absidia* pp), *Apophysomyces, Saksenaea, Cunninghamella, Cokeromyces* y *Syncephalastrum* también producen infecciones en humanos[113]. La mayoría de las infecciones ocurren en inmunodeprimidos, pero también se han descrito casos en pacientes sin factores de riesgo conocidos[115,119]. El mayor aumento de casos se ha registrado en pacientes hematológicos[69,76,106,115]; algunos autores lo han asociado a la introducción de voriconazol como tratamiento de elección de la aspergilosis invasora y como profilaxis en pacientes inmunodeprimidos[63,69,79,128]. Sin embargo, es posible que este incremento se deba también al aumento de la población de riesgo y a una mejora en las herramientas de diagnóstico. Marr et al.[76] detectaron que en el quinquenio 1995-1999 la incidencia de infecciones causadas por hongos mucorales fue el doble que la del quinquenio 1985-1989. La identificación morfológica de los mucorales es, como en el resto de los hongos miceliales, una tarea complicada que exige un gran nivel de conocimiento de la taxonomía. Además, no son infrecuentes los aislados que no esporulan en cultivo y que por tanto, no pueden identificarse mediante sus características morfológicas. En estos casos, para identificar la cepa es imprescindible el uso de herramientas moleculares.

1.3.4. *Scedosporium*

En los últimos años el número de infecciones causadas por las especies del género *Scedosporium* ha aumentado considerablemente por lo que es considerado un hongo emergente[21,70,130,142]. Las especies con mayor relevancia clínica son *Scedosporium apiospermum* (y su teleomorfo *Pseudallescheria boydii*) y *Scedosporium prolificans* que producen una gran variedad de infecciones, desde cutáneas y subcutáneas a diseminadas en individuos inmunodeprimidos. El tratamiento es difícil ya que se trata de

hongos multiresistentes, sobretodo *S. prolificans*, donde las tasas de mortalidad alcanzan el 87,5%[116]. La aplicación de técnicas moleculares ha demostrado que *P. boydii* es un complejo de especies y ha permitido la descripción de algunas especies nuevas como *Scedosporium aurantiacum* y *Scedosporium dehoogii*[42,43].

1.4. Antifúngicos

En los últimos años ha incrementado mucho el número de fármacos disponibles para tratar las infecciones fúngicas. Las especies de hongos capaces de causar infecciones en el hombre, tienen diferentes perfiles de sensibilidad a los antifúngicos. Por tanto, la identificación de las especies y la determinación del perfil de sensibilidad de las mismas se hace imprescindible para elegir la mejor opción terapéutica. En este apartado se van a resumir los antifúngicos que se utilizan actualmente para tratar las infecciones humanas causadas por hongos filamentosos.

1.4.1. Polienos

Los polienos actúan a nivel de la membrana plasmática del hongo uniéndose al ergosterol (Figura 2) y produciendo alteraciones de la permeabilidad con pérdida de contenido citoplasmático y muerte de la célula (efecto fungicida). Aunque existen varios antifúngicos poliénicos, como la nistatina y la natamicina, el más utilizado es la anfotericina B.

Anfotericina B

Durante más de cuarenta años la anfotericina B ha sido el principal y casi único antifúngico para tratar las infecciones fúngicas[39,100]. Tiene un espectro de actividad muy amplio y los casos de resistencia son poco frecuentes, aunque es bien conocida su baja actividad frente a algunos hongos como *A. terreus*[133,143], *P. lilacinus*[17] o las especies del género *Scedosporium*[25,80]. Su principal inconveniente son sus efectos secundarios entre los que destaca una elevada nefrotoxicidad[12]. Con el fin de reducir los efectos adversos se han desarrollado nuevas formulaciones asociadas a complejos lipídicos. Así aparecieron la anfotericina B liposomal, la anfotericina B de dispersión coloidal y la anfotericina B complejo lipídico. Estas nuevas formulaciones poseen una eficacia similar pero tienen menor toxicidad. Sin embargo, su utilización clínica ha tenido poco impacto en prolongar la supervivencia del paciente[33,67,72].

1.4.2. Azoles

La aparición de los imidazoles y posteriormente los triazoles supuso un avance en el tratamiento de las micosis. Estos antifúngicos actúan a nivel de la membrana plasmática

inhibiendo la síntesis del ergosterol (Figura 2). Los primeros azoles (imidazoles) resultan también tóxicos para las células animales, pero el desarrollo de los triazoles disminuyó este problema.

1.4.2.1. Fluconazol

La aparición del fluconazol en los años 90 supuso un gran avance en la terapia antifúngica. Es activo frente a las especies de *Candida* y *Cryptococcus neoformans,* siendo un efectivo tratamiento de primera línea en las candidosis orofaríngeas y esofágicas y para la candidemia[20]. Sin embargo, su espectro de acción es limitado ya que es inactivo frente a la mayoría de los hongos filamentosos y algunas levaduras como *Candida krusei* y *Candida glabrata*[24,106,107].

1.4.2.2. Itraconazol

El espectro de actividad del itraconazol es más amplio que el del fluconazol, siendo activo frente a levaduras y algunos hongos filamentosos, incluyendo especies del género *Aspergillus.* Es útil en la terapia empírica y tratamiento de la candidosis así como las infecciones leves o moderadas de hongos filamentosos. Sin embargo presenta problemas de absorción[109], no es activo frente a los principales patógenos emergentes como *Fusarium, Scedosporium* o mucorales, y algunas especies de *Aspergillus* presentan resistencia[1,10,22].

1.4.2.3. Voriconazol

El voriconazol es un azol de segunda generación cuya estructura química deriva de la del fluconazol[124]. En la actualidad constituye el tratamiento de elección para la aspergilosis invasora[55]. Su espectro de actividad es amplio, además de ser activo frente a *Aspergillus,* también lo es frente a cepas de *Candida* resistentes al fluconazol y algunas especies de los géneros *Scedosporium* y *Fusarium*[45,80]. No tiene actividad frente a los mucorales, siendo varios los autores que han descrito infecciones de brecha por mucorales en pacientes en profilaxis con este antifúngico[79,135,136].

1.4.2.4. Posaconazol

El posaconazol pertenece también a la segunda generación de triazoles, y su estructura es similar a la del itraconazol[123]. Ha sido aprobado para la profilaxis de las infecciones fúngicas invasoras y el tratamiento de la candidosis orofaríngea en pacientes con SIDA. En Europa, además ha sido aprobado para el tratamiento de aspergilosis, fusariosis, cromoblastomicosis y coccidioidomicosis refractarias al tratamiento con anfotericina o itraconazol[122,123]. Es activo frente a todos los hongos que cubren el resto de los triazoles

incluyendo las cepas resistentes[120]. Tiene mayor actividad in vitro, que la anfotericina B y el voriconazol frente a las especies de *Aspergillus*[22,120]. A diferencia del voriconazol es activo frente a los mucorales, aunque no tan activo como la anfotericina B[4,26,46,123]. No obstante, sólo está disponible en formulación oral, lo que dificulta su uso en determinados grupos de pacientes.

1.4.3. Alilaminas

Terbinafina

La terbinafina pertenece a la familia de las alilaminas e interfiere en una de las primeras etapas en la vía de síntesis del ergosterol, haciendo que se acumule en la célula el escualeno lo que conlleva a la ruptura de la membrana y a la muerte celular. Se usa en el tratamiento de las infecciones de uñas y piel, siendo el tratamiento de elección en las onicomicosis. También se ha utilizado en terapia combinada con triazoles para tratar las infecciones causadas por *Scedosporium*[61,130].

1.4.4. Equinocandinas

Las equinocandinas son una familia de antifúngicos que actúan a nivel de la pared celular del hongo, inhibiendo la síntesis del 1,3-β-D-glucano (Figura 2) y produciendo cambios osmóticos que llevan a la lisis celular. Las células humanas no poseen glucano, por lo que la toxicidad es menor que con otros grupos de antifúngicos[16,34,64]. La proporción de β-D-1,3 glucano en la pared celular de los hongos varía mucho entre las distintas especies, siendo más abundante en *Candida* y *Aspergillus*, y estando ausente en los mucorales. Por esta razón, las equinocandinas, tienen actividad fungicida frente a la mayoría de las especies de *Candida* y sin embargo, es fungistática frente a *Aspergillus*. La actividad es muy limitada frente a *Fusarium, Trichosporon, Cryptococcus neoformans* y mucorales[147]. Dentro de esta familia hay tres antifúngicos disponibles: caspofungina, micafungina y anidulafungina.

1.4.4.1. Caspofungina

La caspofungina está aprobada para el tratamiento de la candidosis orofaríngea, esofágica y diseminada, así como para el tratamiento de rescate de la aspergilosis invasora[30,34,74]. Es igual de efectiva que la anfotericina B convencional en el tratamiento de las candidosis pero con menor toxicidad[83]. Podría ser útil como terapia combinada, incluso para el tratamiento de la mucormicosis, ya que ha demostrado potenciar la actividad de la anfotericina B en el tratamiento de estas infecciones[62,111,129].

1.4.4.2. Micafungina

La micafungina fue aprobada por la FDA (Food and Drug Administration en EE.UU.) en 2005 y por la EMEA (European Medicines Agency en la Unión Europea) en 2008. Está indicada para el tratamiento de la candidosis esofágica y la profilaxis en pacientes receptores de trasplante de progenitores hematopoyéticos. Aunque no está aprobada para el tratamiento de la aspergilosis cada vez hay más datos que apoyan su posible efectividad en el manejo de dichas infecciones[32,66]. Estudios recientes sugieren su posible utilidad en terapias combinadas con otros antifúngicos[32,87] lo que aporta una opción más en infecciones refractarias al tratamiento de primera línea.

1.4.4.3. Anidulafungina

La anidulafungina ha sido aprobada por la FDA y por la EMEA para el tratamiento de la candidemia en enfermos no neutropénicos, la candidosis esofágica, abscesos intrabdominales y peritonitis. Es la equinocandina que presenta mayor actividad *in vitro* frente a las especies de *Candida*,[96,105]. Al igual que las otras dos equinocandinas tiene un efecto fungistático frente a *Aspergillus*[125]. Cabe destacar su buena tolerancia, la ausencia de interacciones farmacológicas, y que no es necesario el ajuste de dosis en pacientes con insuficiencia hepática o renal[38]. Por ahora no se ha descrito el desarrollo de resistencias secundarias mientras que sí se han dado casos en caspofungina y micafungina[3,85,145]

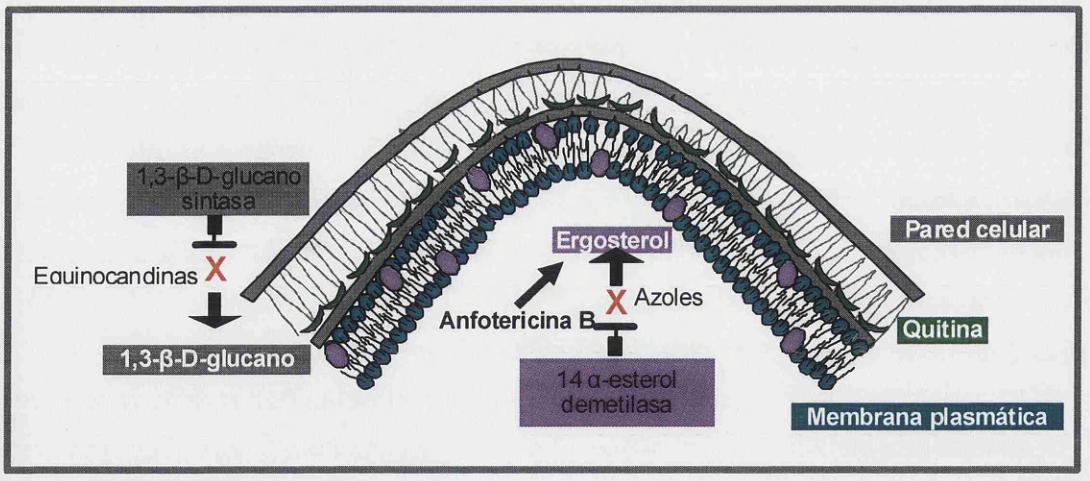


Figura 2: Mecanismo de acción de los antifúngicos

1.5. Hipótesis de trabajo

La hipótesis de partida de este trabajo se basa en la necesidad de desarrollar herramientas de identificación de los principales hongos patógenos humanos y correlacionar esta identificación con el perfil de sensibilidad a los antifúngicos disponibles para tratar dichas infecciones.

2. Objetivos

2. Objetivos

Primero: Analizar la utilidad de la identificación molecular mediante secuenciación de los ITSs y el análisis del perfil de sensibilidad a los antifúngicos de los siguientes grupos de hongos patógenos humanos:

- *Aspergillus:*
    - sección *Fumigati*
    - sección *Nigri*
    - sección *Usti*
- *Fusarium* spp.
- Mucorales
- *Scedosporium* spp.

Segundo: Analizar la taxonomía y los límites de especie en el género *Lichtheimia.*

- Estudios fenotípicos: morfología, crecimiento a diversas temperaturas, test de apareamiento.
- Estudio genotípico: secuenciación de distintos loci.

Tercero: Desarrollar una base de datos de secuencias de ADN y de sensibilidad a los antifúngicos de los principales hongos patógenos humanos, con cepas correctamente identificadas, que permita obtener una identificación rápida y fiable y que ayude a definir el tratamiento más adecuado.

Objectives

First: To evaluate the usefulness of molecular identification by means of ITS sequencing for the main human mould pathogens:

- *Aspergillus*
    - section *Fumigati*
    - section *Nigri*
    - section *Usti*
- *Fusarium* spp.
- Mucorales
- *Scedosporium* spp.

Second: To evaluate species limits and the taxonomy of genus *Lichtheimia.*

- Phenotypic studies: morphology, growth kinetics and mating test
- Molecular study: sequencing of several loci

Third: To develop DNA and susceptibility profile database of the main human fungal pathogens with correctly identified strains, which allow a fast, accurate and trustable identification together with the most adequate treatment.

3. Artículos

3.1. *Aspergillus* spp.

3.1.1. In vitro activity of nine antifungal agents against clinical isolates of *Aspergillus calidoustus*.

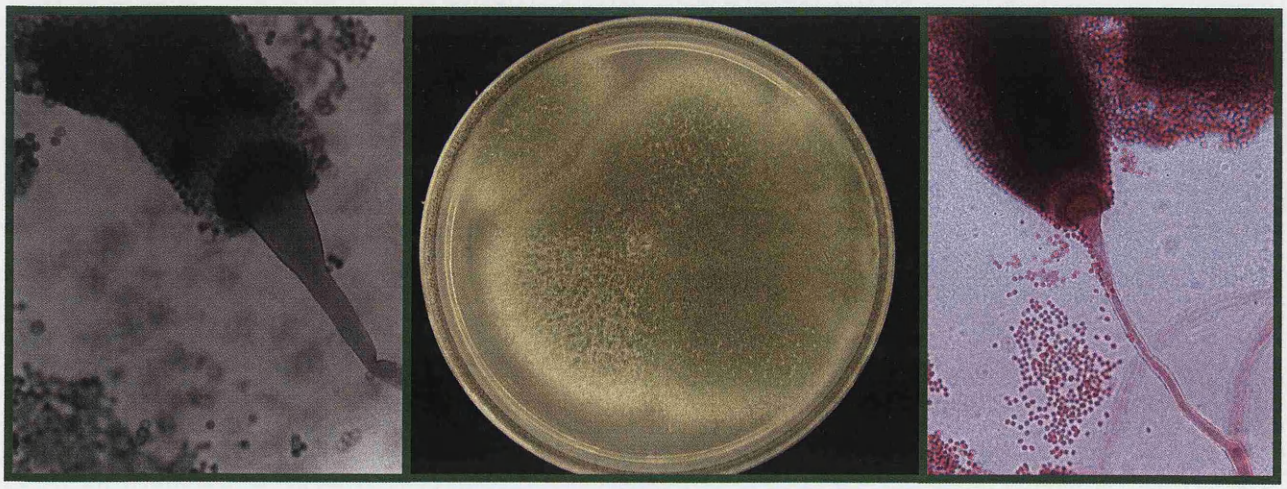
Medical Mycology 2009 Mar 18;1-7

3.1.2. *Aspergillus* Section *Fumigati*: Antifungal Susceptibility Patterns and Sequence-Based Identification.

Antimicrobial Agents and Chemotherapy, Apr. 2008, p. 1244–1251 Vol. 52, No. 4.

3.2.3 Specie identification and Antifungal Susceptibility Patterns of Species Belonging to *Aspergillus* section *Nigri*

Antimicrobial Agents and Chemotherapy 2009 Oct;53(10):4514-7.



3.1.1. *Aspergillus* sección *Usti*

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In vitro activity of nine antifungal agents against clinical isolates of *Aspergillus calidoustus*

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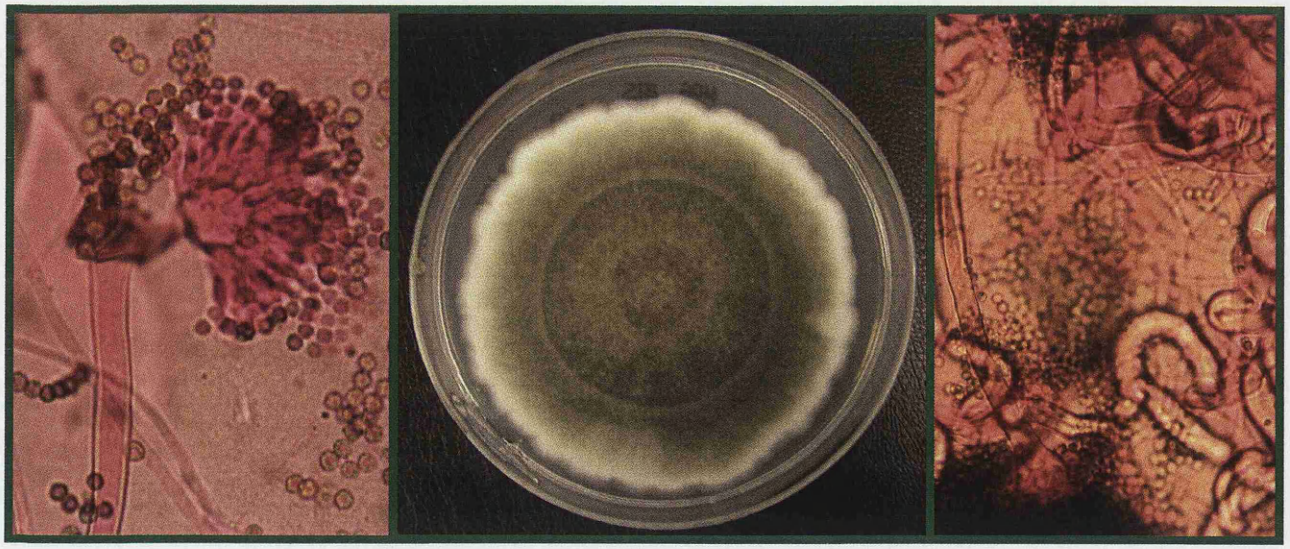
Resumen: Se estudiaron 9 aislados de *Aspergillus* pertenecientes a la colección del Centro Nacional de Microbiología, que fueron identificados morfológicamente como miembros de la sección *Usti*. Las cepas fueron identificadas mediante la secuenciación de la región ITS y parte del gen que codifica la β tubulina. Se identificaron un *Aspergillus pseudodeflectus* y ocho *Aspergillus calidoustus.* Los antifúngicos más activos frente a estas especies fueron terbinafina y las equinocandinas, micafungina y anidulafungina.



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In vitro activity of nine antifungal agents against clinical isolates of *Aspergillus calidoustus*

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This study analyzes *Aspergillus* isolates from the Spanish National Centre for Microbiology collection, which were identified morphologically as members of *Aspergillus* section *Usti*. Strains were identified through the analysis of the Internal Transcribed Spacer regions and partial β tubulin gene sequences. One *Aspergillus pseudodeflectus* isolate and eight *Aspergillus calidoustus* isolates were detected in this panel of clinical strains. Terbinafine and the echinocandins micafungin and anidulafungin, were the drugs most active against these species.

Keywords *Aspergillus calidoustus*, antifungal susceptibility profile, beta tubulin

Introduction

Aspergillus section *Usti* has recently been reviewed on the basis of chemical, molecular and morphological data [1,2]. *Aspergillus insuetus*, which had been synonymized with *Aspergillus ustus* [3], has been revived and is currently a valid species. In addition, two new species have been described in the section *Usti*, i.e., *Aspergillus keveii* and *Aspergillus calidoustus*. Presently, *Aspergillus* section *Usti* includes the following species: *Aspergillus ustus*, *Aspergillus puniceus*, *Aspergillus granulosus*, *Aspergillus pseudodeflectus*, *Aspergillus calidoustus*, *Aspergillus insuetus*, *Aspergillus keveii* and *Emericella heterothallica*.

During recent years there have been several reports on the clinical importance of *A. ustus*. Twenty-four cases have been reported in the literature since 1971, half of them in the last three years [4–7]. Infections due to *A. ustus* have been associated with high mortality rates, and examination of its *in vitro* antifungal susceptibility profile reveals high MIC (minimum inhibitory concentration) values for many drugs [1,2,5,8–10]. Recently, Houbraken *et al.* [1,2] proposed in their taxonomic review of this section that several strains, previously identified as *A. ustus*, should be renamed as *A. calidoustus*.

The internal transcribed spacers (ITS) of the rDNA and a part of the tubulin gene (BenA) were sequenced for all *Aspergillus* isolates from our collection which were morphologically identified as a member of section *Usti*. In addition, *in vitro* antifungal susceptibility testing was performed by means of EUCAST mold methodology [15] to assess their susceptibility profiles.

Material and methods

Strains

Nine strains were analyzed in this investigation, all held at the Mould Collection of the Spanish National Centre for Microbiology (CNM-CM) and previously identified by morphological means as *Aspergillus ustus*. All were obtained from clinical samples, i.e., seven from respiratory sites, one from a vascular biopsy of an aneurysm and one from cerebrospinal fluid. Each isolate was recovered from a different patient and sent to the Spanish Mycology Reference Laboratory for identification and susceptibility testing. Strains were labelled as CNM-CM (Spanish National Centre for Microbiology-moulds culture collection), together with a unique identification number. Three strains were acquired from the culture collection of the CBS-Fungal

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Biodiversity Centre, i.e., two *A. ustus* strains, CNM-CM4036 (CBS 113233) of unknown origin and CNM-CM4037 (CBS 239.90) from a brain abscess and one *A. pseudodeflectus* strain CNM-CM5302 (CBS 756.74) of environmental origin.

Morphological identification

The strains were subcultured on different agar media to ascertain their macroscopic and microscopic morphology. The media included malt extract agar (MEA, 2% malt extract, Oxoid S.A., Madrid, Spain) and Czapek-Dox Agar (Soria Melgizo S.A., Madrid, Spain, Difco 0339-01-1). Cultures were incubated at 30°C and 37°C.

PCR and DNA sequencing of ITS region

Moulds were cultured in GYEP medium (0.3% yeast extract, 1% peptone, Difco, Soria Melguizo S.A) with 2% glucose (Sigma-Aldrich Quimica, Madrid, Spain), for 24 48 h at 30°C. Genomic DNA was isolated using an extraction procedure previously described [11].

DNA segments comprising the ITS1 and ITS2 regions were amplified with primer set ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [12]. Partial sequence of the β-tubulin gene was amplified using primer set βtub3 (5′-TTCACCTTCAGACCGGT-3′) and βtub2 (5′-AGTTGTCGGGACGGAATAG-3′) [13]. All primers were synthesized by Sigma Genosys (Madrid, Spain). The reaction mixtures for ITS amplification contained 0.5 μM of each primer; 0.2 mM of each deoxynucleoside triphosphate (Applied Biosystems, Madrid, Spain); 5 μl of PCR 10 × buffer (Applied Biosystems); 2.5 U *Taq* DNA polymerase (Applied Biosystems); and 25 ng of genomic DNA in a final volume of 50 μl. For the portion of β tubulin, PCRs were run with a volume of 50 μl containing 1 μM of each primer; 250 μM of each deoxynucleoside triphosphate (Applied Biosystems); 1 × PCR buffer (Applied Biosystems); 2 mM $MgCl_2$ (Applied Biosystems); 2.5 U of *Taq* DNA polymerase (Applied Biosystems); and 25 ng of genomic DNA. Amplification was performed in a thermal cycler (GeneAmp PCR system 9 700; Applied Biosystems) using the following cycling parameters: for ITS one initial cycle of 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 45 s at 56°C, and 2 min at 72°C, with one final cycle of 5 min at 72°C; for β tubulin 1 cycle of 5 min at 94°C and then 30 cycles of 30 s at 94°C, 45 s at 55°C, and 1 2 min at 72°C, followed by 1 final cycle of 10 min at 72°C. The PCR products were analyzed by electrophoresis on 0.8% agarose gels and visualized by transillumination (Gel Doc 2000; Bio-Rad Laboratories, Madrid, Spain) after staining with ethidium bromide (Sigma-Aldrich Quimica).

The sequencing reactions were undertaken as described previously [14], with 2 μl of reaction mixture from a sequencing kit (BigDye terminator cycle sequencing kit, Ready Reaction mixture; Applied Biosystems), 1 μM of the primers, and 3 μl of the PCR product in a final volume of 10 μl. The primers used for sequencing were ITS1 and ITS4 for the ITS region and βtub1 (AATTGGTGCCGCTTTCTGG) and βtub4 (5′-AGCGTCCATGGTACCAGG-3′) [13] for the β-tubulin gene. Sequences were assembled and edited using the SeqMan II and EditSeq software packages (Lasergene: DNAstar, Inc., Madison, Wisconsin, USA).

Identification and phylogenetic analysis

Identifications were based on comparisons of the sequences with ITS and β tubulin sequences of *Aspergillus* section *Usti* strains obtained from the GenBank database. The phylogenetic analyses were conducted with InfoQuest FP software, version 4.50 (BIORAD Laboratories, Madrid, Spain). The methodology used was maximum parsimony clustering. Phylogram stability was assessed by parsimony bootstrapping with 2000 simulations. *Aspergillus fumigatus* CNM-CM237 was used as an outgroup to root the phylograms.

GenBank accession numbers

Following are the GenBank accession numbers for β tubulin fragment sequence from the strains included in this work: CBS121601 FJ624456; CNM-CM2105 FJ624457; CNM-CM2272 FJ624458; CNM-CM237 FJ624459; CNM-CM2475 FJ624460; CNM-CM3788 FJ624461; CNM-CM3927 FJ624462; CNM-CM4036 FJ624463; CNM-CM4037 FJ624464; CNM-CM4115 FJ624465; CNM-CM4212 FJ624466; CNM-CM5070 FJ624467

Antifungal susceptibility testing

Microdilution testing was performed following the EUCAST standard methodology [15 18]. *Aspergillus fumigatus* ATCC 2004305 and *Aspergillus flavus* ATCC 2004304 were used as quality control strains [19].

The following antifungal agents were tested: Amphotericin B (Sigma-Aldrich Quimica), itraconazole (Janssen Pharmaceutica, Madrid, Spain); voricon azole (Pfizer S.A., Madrid, Spain); ravuconazole (Bristol-Myers Squibb, Princeton, USA); posaconazole (Schering-Plough Research Institute, Kenilworth, NJ); terbinafine (Novartis, Basel, Switzerland); caspofungin (Merck & Co., Inc., Rahway, NJ); micafungin (Astellas

pharma Inc., Tokio, Japan) and anidulafungin (Pfizer S.A.). The final concentrations tested range from 16 to 0.03 mg/l for amphotericin B, terbinafine, caspofungin and anidulafungin, and from 8 to 0.015 mg/l for itraconazole, voriconazole, ravuconazole and posaconazole. The plates were incubated at 35°C for 48 h in a humid atmosphere. Visual readings were performed with the help of a mirror. The endpoint for amphotericin B, itraconazole, voriconazole, ravuconazole, posaconazole and terbinafine was defined as the antifungal concentration that produced a complete inhibition of visual growth at 48 h. For the echinocandins the endpoint was the antifungal concentration that produced a visible change in the morphology of the hyphae as compared with the growth in control well (minimum effective concentration, MEC) [20,21]. The antifungal susceptibility testing was repeated at least three times on different days for each isolate.

Results

Identification of strains

Morphological examination showed that all nine isolates were members of *Aspergillus* section *Usti*. All grew at 30 and 37°C in contrast with the *A. ustus* strains from the CBS culture collection,which were not able to grow at 37°C.

The identification to species level was assessed by molecular methods, analyzing the ITS regions (data not shown) and partial β tubulin gene sequences (Fig. 1). Eight out of nine strains were identified as *A. calidoustus* and one as *A. pseudodeflectus* (CNM-CM5070). No other species were found in this collection of clinical isolates. The β tubulin sequence was the only characteristic that differed between *A. calidoustus* and *A. pseudodeflectus*. Both species shared the same ITS sequence and are able to grow at 37°C.

Antifungal susceptibility testing

The geometric mean (GM) and range of the MICs of antifungal agents for each isolate is shown in Table 1. Due to the limited number of isolates, a susceptibility pattern could not be obtained. In any case, anidulafungin and micafungin have excellent activity *in vitro* against all isolates shown in Table 1. Amphotericin B also shows significant activity *in vitro* against the clinical isolates, with the exception of one *A. ustus* and one *A. calidoustus* isolate. Finally, only one *A. pseudodeflectus* isolate showed high MICs against terbinafine.

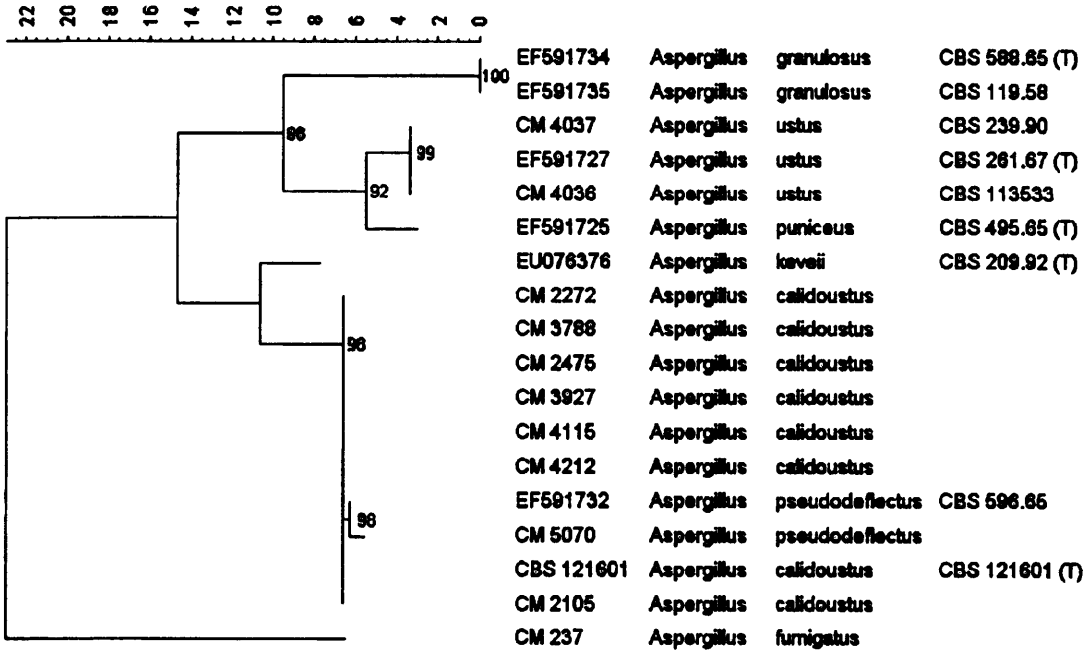

Fig. 1 Phylogenetic tree of the subset of isolates included in the study obtained by using maximum parsimony phylogenetic analyses and 2000 bootstrap simulations based on a portion of the β tubulin sequences. *Aspergillus fumigatus* CNM-CM237 was used as an outgroup to root the tree.

Table 1 Geometric mean (GM) and range of MICs (mg/l) of antifungal agents tested in the study.

	Amphotericin B		Itraconazole		Voriconazole		Ravuconazole		Posaconazole		Terbinafine		Caspofungin		Micafungin		Anidulafungin	
	GM	Range	GM	Range	GM	Range	GM	Range	GM	Range	GM	Range	GM	Range	GM	Range	GM	Range
A. calidoustus																		
CNM-CM1709	1	0.5–2	16	16–16	8	8–8	8	8–8	12.699	8–16	0.63	0.25–2	3.175	2–8	0.122	0.03–0.5	0.019	0.015–0.03
CNM-CM 2105	1	0.5–2	16	16–16	8	8–8	3.175	2–4	16	16–16	0.5	0.25–1	3.175	2–4	0.038	0.015–0.120	0.019	0.015–0.03
CNM-CM 2272	0.794	0.5–1	16	16–16	8	8–8	4	4–4	12.699	8–16	0.397	0.25–0.5	6.35	4–8	0.122	0.03–0.5	0.019	0.015–0.03
CNM-CM 2475	1	0.5–2	16	16–16	8	8–8	2	2–2	16	16–16	0.5	0.25–1	8	8–8	0.015	0.015–0.015	0.015	0.015–0.015
CNM-CM 3788	0.5	0.25–1	16	16–16	6.35	4–8	3.175	2–4	12.699	8–16	0.5	0.25–1	0.63	0.5–1	0.077	0.015–1	0.021	0.015–0.03
CNM-CM 3927	1.578	1–2	12.699	8–16	4	4–4	3.175	2–4	10.079	4–16	0.315	0.25–0.5	0.794	0.5–2	0.038	0.015–0.12	0.019	0.015–0.03
CNM-CM 4115	0.707	0.5–1	16	16–16	8	8–8	4	4–4	16	16–16	0.63	0.5–1	3.175	2–4	0.077	0.015–0.25	0.024	0.015–0.06
CNM-CM 4212	1.26	1–2	16	16–16	8	8–8	4	4–4	10.079	4–16	0.315	0.25–2	10.079	2–32	0.048	0.015–0.25	0.06	0.015–0.12
GM	0.93		15.54		7.13		3.67		13.07		0.46		3.08		0.06		0.02	
A. ustus																		
CNM-CM 4036	6.35	4–8	16	16–16	1.26	1–2	0.5	0.25–1	4	2–16	0.315	0.25–0.5	3.175	2–4	0.03	0.015–0.06	0.019	0.015–0.03
CNM-CM 4037	1.26	1–2	1.26	0.5–2	1.26	1–2	0.5	0.25–1	2	1–4	0.153	0.12–0.25	4	4–4	0.123	0.03–0.25	0.038	0.015–0.12
GM	2.83		4.49		1.26		0.50		2.33		0.22		3.56		0.06		0.03	
A. pseudodeflectus																		
CNM-CM 5070	0.25	0.12–0.5	16	16–16	8	8–8	8	8–8	16	16–16	0.5	0.25–1	4	2–8	0.125	0.03–0.5	0.015	0.015–0.015
CNM-CM 5302	10.1	4–32	16	16–16	16	16–16	8	8–8	16	16–16	8	8–16	0.25	0.12–0.5	0.015	0.015–0.015	0.015	0.015–0.015
GM	1.59		16		11.3		8		16		2		1		0.04		0.02	

Activity of azole drugs was very limited against *A. calidoustus* and *A. pseudodeflectus* isolates. However, voriconazole and ravuconazole showed good activity against both *A. ustus* isolates.

Discussion

Non-*Aspergillus fumigatus Aspergillus* species are increasingly isolated from human samples. An interim analysis of the TransNet surveillance program showed that *A. fumigatus* caused 56% of cases of invasive aspergillosis after hematopoietic stem cell transplantation [23]. Gomez-Lopez *et al.* [24] reported a prevalence of 45.4% of this species in a study with 338 clinical samples.

Houbraken *et al.* [1] and Varga *et al.* [2] have recently reviewed the taxonomy of *Aspergillus* section *Usti*, describing new species based on a combination of morphology, physiology, extrolite patterns and molecular techniques. *Aspergillus calidoustus* has been described as the species most commonly associated with human beings. This species forms a well-defined clade with *A. pseudodeflectus* in their ITS sequence but could be differentiated from this specie by analyzing a portion of the β tubulin gene. Both species are able to grow at 37°C.

In recent years infections caused by *A. ustus* have increased significantly. This species has been reported as the agent of a number of different infections such as endocarditis, pneumonia, primary cutaneous infections, invasive pulmonary aspergillosis and endophthalmitis. Varga *et al.* [2] included *A. ustus* strains used in a previously reported paper by Panakal *et al.* [4] in their study, and found that these strains could be identified as *A. calidoustus*. This fact concurred with our results, where no *A. ustus* was isolated from clinical samples, and therefore allows us to assume that most of the clinical cases of *A. ustus* reported are due to *A. calidoustus*. In addition, one *A. pseudodeflectus* isolate was present among the analyzed strains. This species is rarely encountered but the limited reports of its occurrence could be due to the fact of it having been misidentified in the literature as *A. ustus*.

MICs obtained from different groups showed low activities *in vitro* for most of the drugs tested [4,5,10]. Since 1974, 24 infections caused by *A. ustus* have been described in the literature. Amphotericin B, caspofungin, voriconazole, itraconazole and combination therapies have been used to treat these infections with a low rate response. Eight therapy successes have been described [4,5,25 27], but only four patients survived.

This study reports the susceptibility data of nine Spanish clinical isolates and three CBS strains, identified by morphological and molecular methods. Despite the fact that the number of isolates is limited, practical recommendations can be obtained from the data. It seems that *A. calidoustus* is the most frequent species of section *Usti* associated with humans. At a time when prophylaxis with azole drugs is standard practice, for certain populations of patients these resistant species could be selected, as azole drugs are not effective against them.

Susceptibility testing *in vitro* showed that terbinafine and the echinocandins, micafungin and anidulafungin were the most active drugs against *A. calidoustus, A. ustus* and *A. pseudodeflectus*. Other authors have reported similar results for terbinafine [4,10]. However, no data has been found so far on *in vitro* activity of micafungin and anidulafungin. These two new echinocandins can be useful for the treatment of these fungi, as the *in vitro* susceptibility testing has shown. In addition, this study as well as another report [4], have shown effective action of amphotericin B against this group of fungi (Table 1). In summary, identification at species level for *Aspergillus* species has become an important issue since species with high *in vitro* MICs have been increasingly involved in fungal infections. *A. calidoustus* is a recently described, multi-resistant fungus that can be found in clinical samples, and can be accurately identified at species level by sequencing a part of the β tubulin gene. Optimal treatment for these infections has not been established yet. Susceptibility testing should therefore be performed in order to provide alternatives for the treatment of this infection.

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Madrileña Foundation. He has been an advisor/consultant to the Panamerican Health Organization, Gilead Sciences, Merck Sharp and Dohme, Pfizer, and Schering Plough. He has been paid for talks on behalf of Gilead Sciences, Merck Sharp and Dohme, Pfizer, and Schering Plough.

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Other authors: no conflicts

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3.1.2. *Aspergillus* sección *Fumigati*

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Aspergillus Section *Fumigati*: Antifungal Susceptibility Patterns and Sequence-Based Identification[▽]

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Resumen: Este estudio analizaron 28 cepas de *Aspergillus* pertenecientes a la sección *Fumigati*, que fueron aisladas de muestras clínicas en España. Todos los aislados esporularon lentamente y no crecieron a 48 °C. La identificación molecular se llevó acabo mediante la secuenciación de una parte de los genes que codifican para la β tubulina y el rodlet A, identificándose seis especies diferentes (*Neosartorya hiratsukae, Neosartorya pseudofischeri, Aspergillus viridinutans, Aspergillus lentulus, Aspergillus fumigatiaffinis* y *Aspergillus fumisynnematus).* Los perfiles de sensibilidad fueron heterogéneos. La mayoría de los aislados de *A. lentulus* y *A. fumigatiaffinis* mostraron CMIs elevadas a anfotericina B (media geométrica [MG] CMIs, >4.5 mg/L), itraconazol (MG CMIs, >6 mg/L), voriconazol (MG CMIs, >3 mg/L) y ravuconazol (MG CMIs, >3 mg/L); *N pseudofischeri* y *A. viridinutans* mostraron CMIs elevadas a itraconazol (MG CMIs, >8 mg/L), voriconazol (MG CMIs, >3.33 mg/L) y ravuconazol (MG CMIs, >2 mg/L); y *N. hiratsukae* y *A. fumisynnematus* fueron sensibles a todos los antifúngicos ensayados. En conclusión, varias especies que morfológicamente se parecen a *A. fumigatus* pueden causar infecciones invasoras en humanos. Además, algunas de ellas tienen CMIs altas para la mayoría de los antifúngicos disponibles para el tratamiento de los pacientes infectados con *Aspergillus.* La epidemiología y la relevancia clínica de estas especies debe ser estudiada con más detenimiento.

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This study analyzed 28 *Aspergillus* strains belonging to the section *Fumigati* that were isolated from clinical samples in Spain. All isolates sporulated slowly and were unable to grow at 48°C. Phylogenetic analysis based on sequencing of partial sequences of the β-tubulin and rodlet A genes was used to classify the 28 strains into six different clades (*Neosartorya hiratsukae*, *Neosartorya pseudofischeri*, *Aspergillus viridinutans*, *Aspergillus lentulus*, *Aspergillus fumigatiaffinis*, and *Aspergillus fumisynnematus*). Antifungal susceptibility testing showed heterogeneous patterns and grouped the strains together by species. Most *A. lentulus* and *A. fumigatiaffinis* isolates showed high MICs of amphotericin B (geometric mean [GM] MICs, ≥4.5 μg/ml), itraconazole (GM MICs, ≥6 μg/ml), voriconazole (GM MICs, ≥3 μg/ml), and ravuconazole (GM MICs, ≥3 μg/ml); *N. pseudofischeri* and *A. viridinutans* showed high MICs of itraconazole (GM MICs, ≥8 μg/ml), voriconazole (GM MICs, ≥3.33 μg/ml), and ravuconazole (GM MICs, ≥2 μg/ml); and *N. hiratsukae* and *A. fumisynnematus* were susceptible to all the antifungals tested. In conclusion, a number of different species whose morphological features resemble those of *Aspergillus fumigatus* could succeed in producing invasive infections in the susceptible host. In addition, some of them showed high MICs for most of the antifungals available for the treatment of patients infected with these organisms. The epidemiology and clinical relevance of these species should therefore be addressed.

The incidence of invasive aspergillosis continues to increase, due to the rising number of patients undergoing bone marrow or solid organ transplantation or corticosteroid treatment and those with hematological malignancies or pulmonary disease (18).

Invasive aspergillosis is mainly caused by *Aspergillus fumigatus*, although other species, such as *Aspergillus terreus*, *Aspergillus niger*, and *Aspergillus flavus* can also cause invasive infections (10, 15, 22). Furthermore, recent studies have reported on cases of aspergillosis caused by other *Aspergillus* species that belong to *Aspergillus* section *Fumigati* (4, 12, 14, 17).

Aspergillus section *Fumigati* has recently been reclassified by Samson et al. (36). It currently contains 25 different species, with 8 anamorphs (*Aspergillus brevipes*, *Aspergillus duricaulis*, *A. fumigatus*, *Aspergillus fumigatiaffinis*, *Aspergillus lentulus*, *Aspergillus novofumigatus*, *Aspergillus unilateralis*, *Aspergillus viridinutans*) and 17 telemorphs (*Neorsartorya aurata*, *Neorsartorya aureola*, *Neorsartorya coreana*, *Neorsartorya fennelliae*, *Neorsartorya fischeri*, *Neorsartorya glabra*, *Neorsartorya lacinosa*, *Neorsartorya spinosa*, *Neorsartorya quadricincta*, *Neorsartorya stramenia*, *Neorsartorya spathulata*, *Neorsartorya hiratsukae*, *Neorsartorya pseudofischeri*, *Neorsartorya tetenoi*, *Neorsartorya mulplicata*, *Neorsartorya udagawae*, and *Neorsartorya sublevispora*).

In the section *Fumigati*, besides *A. fumigatus*, other species, such as *Neorsartorya fischeri*, *Neorsartorya pseudofischeri*, *Neorsartorya hiratsukae*, and *A. lentulus*, have been reported to be human pathogens (3, 4, 12, 14, 17). This implies that in the appropriate human host, all of them could cause disease.

The conventional means of identification of *A. fumigatus* is based on its morphological characteristics and microscopic features. Several morphological characteristics for differentiation between species of the *Aspergillus* section *Fumigati* have been described. However, many species sporulate very slowly, and an extremely high level of expertise and long-term observation are also required to identify the species. Other species, such as those of the genus *Neosartorya*, are able to produce ascospores; however, a considerable length of time is usually required for the production of ascospores, and ascospore production is not a practical method of identification for clinical microbiology laboratories (14, 16, 17, 36). Although for the very experienced taxonomist the species included in the section *Fumigati* are not morphologically uniform, morphological observation is not sufficient to distinguish between them. This fact has led to species misidentification and also to the discarding of organisms as contaminants (6, 44).

In order to resolve this issue, a number of different techniques have been developed and used to identify the species belonging to this section. These include analysis of the profiles of secondary metabolites, isozyme electrophoretic pattern analysis (23, 27, 33, 38, 39, 40), and molecular data analysis (41, 44).

Although secondary resistance to azole drugs has been described in *A. fumigatus* strains (7, 8, 11, 25, 26, 28, 42), most *A. fumigatus* strains are susceptible to the antifungals available for the treatment of patients with invasive infections (13). Because few clinical laboratories routinely perform antifungal susceptibility testing of molds and resistance in the section *Fumigati* has already been reported (5, 24), the misidentification of these species is a matter of concern.

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TABLE 1. Origin, molecular identification, MICs, and MECs of different antifungal against clinical isolates of *Aspergillus* section *Fumigati*

Isolate	Molecular identification			MIC (μg/ml)[a]						MEC (μg/ml)[b]	
	Origin[c]	β-Tubulin	Rodlet A	AMB	ITC	VCZ	RVC	POS	TRB	CAS	MICA
ATCC 2004305	Reference strain	*A. fumigatus*	*A. fumigatus*	0.25–1.0	0.125–1.0	0.25–1.0	0.25–1.0	0.03–0.25	2.0–8.0	0.12–0.5	0.03
CNM-CM-1290	Sputum	*A. lentulus*	*A. lentulus*	4.5	9.25	4.5	3.5	0.40	1.33	0.28	0.05
CNM-CM-3134	Sputum	*A. lentulus*	*A. lentulus*	10	10.25	6	6	0.56	1.75	0.38	0.07
CNM-CM-3364	BAL	*A. lentulus*	*A. lentulus*	8.4	8	5.6	5.6	2	0.5	0.18	0.03
CNM-CM-3537	Sputum	*A. lentulus*	*A. lentulus*	7	8.25	3.5	3	0.28	1	0.42	0.04
CNM-CM-3538	Sputum	*A. lentulus*	*A. lentulus*	7.25	8.25	7.5	7	0.75	1	0.11	0.10
CNM-CM-3583	Sputum	*A. lentulus*	*A. lentulus*	8	0.43	3.5	2	0.25	1.25	1	0.03
CNM-CM-3599	Sputum	*A. lentulus*	*A. lentulus*	2.66	0.5	4	2	0.5	1	0.26	0.03
CNM-CM-4330	Sputum	*A. lentulus*	*A. lentulus*	8	6	3	1.5	0.12	0.5	1.2	0.03
CNM-CM-4370	Sputum	*A. lentulus*	*A. lentulus*	7.33	8	5.33	5.33	0.18	0.5	0.91	0.03
CNM-CM-4387	Sputum	*A. lentulus*	*A. lentulus*	6	8	4	3	0.25	0.5	0.25	0.03
CNM-CM-4415	Sputum	*A. lentulus*	*A. lentulus*	6	16	5.33	4.66	0.33	0.5	0.25	0.03
CNM-CM-4420	Nail	*A. lentulus*	*A. lentulus*	10.66	8	5.33	4	0.33	1	0.33	0.03
CNM-CM-4426	Sputum	*A. lentulus*	*A. lentulus*	12	6.66	5.33	5.33	0.20	0.83	0.22	0.03
CNM-CM-4428	Skin	*A. lentulus*	*A. lentulus*	12	7	5	5	0.62	0.66	0.29	0.03
CNM-CM-4063	BAS	*A. fumisynnematus*	*A. fumisynnematus*	1	0.25	0.83	0.83	0.12	1.66	0.5	0.03
CNM-CM-2280	Sputum	*A. fumigatiaffinis*	*A. fumigatiaffinis*	16	6.66	6.66	6	1.16	1.66	1.3	0.04
CNM-CM-3227	BAL	*A. fumigatiaffinis*	*A. fumigatiaffinis*	8	8	5.6	5	0.625	1.4	0.16	0.08
CNM-CM-3303	Skin	*N. hiratsukae*	*N. hiratsukae*	0.5	0.12	0.5	0.375	0.06	0.09	0.34	0.03
CNM-CM-3305	Skin	*N. hiratsukae*	*N. hiratsukae*	0.31	0.185	0.375	0.25	0.045	0.06	0.03	0.14
CNM-CM-3764	OPE	*N. hiratsukae*	*N. hiratsukae*	1	0.33	1.66	1.33	0.16	0.25	0.26	0.04
CNM-CM-3740	OPE	*N. hiratsukae*	*N. hiratsukae*	0.66	0.16	1	0.66	0.10	0.20	0.66	0.03
CNM-CM-4328	Corneal	*N. hiratsukae*	*N. hiratsukae*	0.75	0.25	1.25	0.5	0.09	0.09	0.03	0.03
CNM-CM-3769	Sputum	*N. pseudofischeri*	*N. pseudofischeri*	1	8	3.33	2	0.25	1.33	0.12	0.06
CNM-CM-2270	Sputum	*N. pseudofischeri*	*N. pseudofischeri*	0.17	8	3	3.5	0.31	1	0.51	0.26
CNM-CM-4060	Sputum	*N. pseudofischeri*	*N. pseudofischeri*	0.25	11	3.33	3.33	0.29	0.5	0.62	0.03
CNM-CM-3914	Nail	*N. pseudofischeri*	*N. pseudofischeri*	0.25	16	6.66	4	0.5	0.5	1	0.03
CNM-CM-3147	OPE	*A. viridinutans*	*A. viridinutans*	0.58	14.4	4	5.66	0.41	1.2	0.67	0.20
CNM-CM-4518	Nail	*A. viridinutans*	*A. viridinutans*	0.37	16	4	4	0.25	0.75	1	0.03

[a] The MICs of amphotericin B (AMB), itraconazole (ITC), voriconazole (VCZ), ravuconazole (RVC), posaconazole (POS), and terbinafine (TRB) are GMs. The MICs for the reference strain are ranges.
[b] The MECs of caspofungin (CAS) and micafungin (MICA) are GMs. The CAS MEC for the reference strain is the range.
[c] BAL, bronchoalveolar lavage fluid; BAS, bronchoalveolar aspirate; OPE, oropharyngeal exudate.

The aim of this study was to analyze clinical strains of *Aspergillus* section *Fumigati*. To date, we have analyzed a collection of 28 *Aspergillus* clinical strains that had previously been identified as atypical *A. fumigatus* isolates. We report on the results of the molecular identification by sequencing of both the β-tubulin and the rodlet A genes and the antifungal susceptibility testing profiles of the strains.

MATERIALS AND METHODS

Fungal strains and media. A total of 37 strains were included in this study; 28 *Aspergillus* section *Fumigati* strains (Table 1) were independent clinical isolates from different patients and belong to the Mold Collection of the Centro Nacional de Microbiología (CNM). Control strains included a set of five *A. fumigatus* strains belonging to the CNM (strains CNM-CM-3248, CNM-CM-3254, CNM-CM-3258, CNM-CM-3652, and CNM-CM-3722) and four strains obtained from the Centraalbureau voor Schimmelcultures (CBS) (*N. hiratsukae* CNM-CM-4551 [CBS 109356] and CNM-CM-4554 [CBS 117067] and *N. pseudofischeri* CNM-CM-4487 [CBS 404.67] and CNM-CM-4488 [CBS 208.92]).

The fungi were grown at 37°C in potato dextrose agar (Oxoid, Madrid, Spain) or malt extract agar. The conidial stocks were preserved in sterile distilled water at 4°C (2).

Fungal morphology and growth conditions. Fungal morphological features were examined by conventional methods (9). The differential temperature growth was determined by the presence or absence of growth at 37°C and 48°C for 3 days (4).

Antifungal susceptibility testing. Broth microdilution susceptibility testing was performed as described in the CLSI (formerly NCCLS) reference method (29), with minor modifications. The modifications included the use of RPMI 1640 with L-glutamine buffered to pH 7 with 0.165 M morpholinepropanesulfonic acid and 10 M NaOH and supplemented with 2% glucose (Oxoid) and the use of an inoculum size of 1×10^5 to 5×10^5 CFU/ml (20, 31, 34).

The antifungal agents used in the study were amphotericin B (concentration range, 16 to 0.03 μg/ml; Sigma Aldrich Química), itraconazole (concentration range, 8 to 0.015 μg/ml; Janssen S.A., Madrid, Spain), voriconazole (concentration range, 8 to 0.015 μg/ml; Pfizer, S.A.), ravuconazole (concentration range, 8 to 0.015 μg/ml; Bristol-Myers, Squibb, Princeton, NJ), posaconazole (concentration range, 8 to 0.015 μg/ml; Schering-Plough Research Institute, Kenilworth, NJ), terbinafine (concentration range, 16 to 0.03 μg/ml; Novartis, Basel, Switzerland), caspofungin (concentration range, 16 to 0.03 μg/ml; Merck & Co, Inc., Rahway, NJ), and micafungin (concentration range, 16 to 0.03 μg/ml; Astellas Pharma Inc, Tokyo, Japan). Inoculum suspensions were prepared from fresh, mature (3- to 5-day-old) cultures by the use of a previously reported methodology (34). The plates were incubated at 35°C for 48 h in a humid atmosphere. Visual readings were performed with the help of a mirror. The endpoint for the MIC determination was the antifungal concentration that produced the complete inhibition of visual growth at 48 h. For the echinocandins, the minimal effective concentration (MEC) was used for endpoint determination. The MEC was defined as the minimal antifungal concentration that produced morphological alterations of hyphal growth at 48 h.

A. fumigatus ATCC 2004305 was used as the quality control strain to validate the MICs and MECs (reference values are shown in Table 1). Antifungal susceptibility testing was repeated at least three times on different days.

PCR amplification and sequencing. Partial sequences of the β-tubulin and the rodlet A genes were amplified with primer set βtub3 (5′-TTCACCTTCAGAC CGGT-3′) and βtub2 (4) and primer set RodA1 and RodA2, respectively (4).

All primers were synthesized by Sigma Genosys (Madrid, Spain). PCRs were carried out with a 50-μl volume containing 1× PCR buffer (Applied Biosystems, Madrid, Spain); 2 mM $MgCl_2$ (Applied Biosystems); 250 μM each of dATP, dGTP, dCTP, and dTTP (Applied Biosystems); 1 μM of each primer; 2.5 U of *Taq* DNA polymerase (Applied Biosystems); and 25 to 50 ng of *A. fumigatus* genomic DNA. Amplification was performed in a thermal cycler (GeneAmp PCR system 9700; Applied Biosystems) for 1 cycle of 5 min at 94°C and then 30 cycles of 30 s at 94°C, 45 s at 55°C, and 1 to 2 min at 72°C, followed by 1 final cycle of 10 min at 72°C. The PCR products were analyzed by electrophoresis on 0.8% agarose gels and were visualized by transillumination (Gel Doc 2000; Bio-Rad Laboratories, Madrid, Spain) after they were stained with ethidium bromide (Sigma, Madrid, Spain).

The sequencing reactions were undertaken as described before (1) with primers βtub1 (4) and βtub4 (5′-AGCGTCCATGGTACCAGG-3′) for the β-tubulin gene and primers RodA3 (5′-AACGTCCGCTTCCCCGTTC-3′), RodA4 (5′-TACGGCATCGGAAGGAGAG-3′), and RodA5 (5′-TACGGCATCGGAGGG AGAG-3′) for the rodlet A gene.

TABLE 2. GenBank sequences and accession numbers of the genes used in this study

GenBank accession no.	Gene	Isolate
DQ094884	β-Tubulin	*A. fumigatiaffinis* IBT 13131
DQ094885	β-Tubulin	*A. fumigatiaffinis* IBT 12703
AB248076	β-Tubulin	*A. fumisynnematus* IFM 42277
AB249897	Rodlet A	*A. fumisynnematus* IFM 42277
AB248077	β-Tubulin	*A. fumisynnematus* 90-BP-70
AB249898	Rodlet A	*A. fumisynnematus* 90-BP-70
AB248078	β-Tubulin	*A. fumisynnematus* 90-BP-177
AB249899	Rodlet A	*A. fumisynnematus* 90-BP-177
AY738513	β-Tubulin	*A. lentulus* FH 5
AY738514	Rodlet A	*A. lentulus* FH 5
AY738517	β-Tubulin	*A. lentulus* FH 4
AY738519	Rodlet A	*A. lentulus* FH 4
AY738520	β-Tubulin	*A. lentulus* FH 7
AY738522	Rodlet A	*A. lentulus* FH 7
AY738523	β-Tubulin	*A. lentulus* FH 220
AY738525	Rodlet A	*A. lentulus* FH 220
DQ094886	β-Tubulin	*A. novofumigatus* IBT 16806
DQ094887	β-Tubulin	*A. novofumigatus* IBT 16755
AB248299	β-Tubulin	*A. viridinutans* IFM 54303
AY590130	β-Tubulin	*A. viridinutans* MK284
AB250103	Rodlet A	*A. viridinutans*
AF057312	β-Tubulin	*A. clavatus* H 522
AF057322	Rodlet A	*A. clavatus* H 522

Sequence analysis. The sequences were assembled and edited with the SeqMan II and EditSeq software packages (Lasergene; DNAStar, Inc., Madison, WI). Sequence analysis was performed by comparing the DNA sequences with those of the control strains included in this study and with the sequences obtained from the GenBank database. Fourteen β-tubulin gene partial sequences and nine rodlet A gene partial sequences were used and are listed in Table 2.

Phylogenetic analysis. All phylogenetic analyses were conducted with InfoQuest FP software (version 4.50; Bio-Rad Laboratories). The methodology used was maximum-parsimony clustering. Phylogram stability was assessed by parsimony bootstrapping with 2,000 simulations. The *Aspergillus clavatus* β-tubulin and rodlet A gene sequences were used as the outgroups (Table 2).

Nucleotide sequence accession numbers. The following are the GenBank accession numbers for the β-tubulin and rodlet A gene fragment sequences from all the strains used in this work: for the β-tubulin gene fragments, CM-1290, EU310839; CM-2270, EU310840; CM-2280, EU310841; CM-3134, EU310842; CM-3147, EU310843; CM-3227, EU310844; CM-3248, EU310845; CM-3254, EU310846; CM-3258, EU310847; CM-3303, EU310848; CM-3305, EU310849; CM-3364, EU310850; CM-3537, EU310851; CM-3538, EU310852; CM-3583, EU310853; CM-3599, EU310854; CM-3652, EU310855; CM-3722, EU310856; CM-3740, EU310857; CM-3764, EU310858; CM-3769, EU310859; CM-3914, EU310860; CM-4060, EU310861; CM-4063, EU310862; CM-4328, EU310863; CM-4330, EU310864; CM-4370, EU310865; CM-4387, EU310866; CM-4415, EU310867; CM-4420, EU310868; CM-4426, EU310869; CM-4428, EU310870; and CM-4518, EU310871; for the rodlet A gene fragments, CM-1290, EU310806; CM-2270, EU310807; CM-2280, EU310808; CM-3134, EU310809; CM-3147, EU310810; CM-3227, EU310811; CM-3248, EU310812; CM-3254, EU310813; CM-3258, EU310814; CM-3303, EU310815; CM-3305, EU310816; CM-3364, EU310817; CM-3537, EU310818; CM-3538, EU310819; CM-3583, EU310820; CM-3599, EU310821; CM-3652, EU310822; CM-3722, EU310823; CM-3740, EU310824; CM-3764, EU310825; CM-3769, EU310826; CM-3914, EU310827; CM-4060, EU310828; CM-4063, EU310829; CM-4328, EU310830; CM-4330, EU310831; CM-4370, EU310832; CM-4387, EU310833; CM-4415, EU310834; CM-4420, EU310835; CM-4426, EU310836; CM-4428, EU310837; and CM-4518, EU310838.

RESULTS

Clinical isolate morphology and growth temperature. The clinical origins of the 28 *Aspergillus* section *Fumigati* strains are shown in Table 1. These were isolated from 21 respiratory specimens, 1 ocular swab specimen, and 6 skin or nail samples.

All strains were first identified as atypical *A. fumigatus* strains by conventional macroscopic and microscopic morphological analysis methods. Identification to the *Aspergillus* genus level was straightforward; however, we were not able to discriminate between the different species of *Aspergillus* section *Fumigati*. While *A. fumigatus* grew at 37°C and 48°C, all the other strains analyzed in this study grew at 37°C but were not able to grow at 48°C.

Molecular identification of *Aspergillus* section *Fumigati*. Partial DNA sequences of the β-tubulin and rodlet A genes were obtained and analyzed. Four CBS strains were amplified with the primers described above in order to compare the 28 atypical *Aspergillus* with members of the section *Fumigati*. We also included a set of β-tubulin and rodlet A sequences from members of *Aspergillus* section *Fumigati* available from GenBank (Table 2).

The phylogenetic tree produced by maximum parsimony of the β-tubulin sequences (Fig. 1) grouped the 28 clinical isolates into six different clades (*N. hiratsukae*, *N. pseudofischeri*, *A. viridinutans*, *A. lentulus*, *A. fumigatiaffinis*, and *A. fumisynnematus*).

On the basis of these results, 14 atypical *A. fumigatus* strains were identified as *A. lentulus*, supported by bootstrap values from 70% to 99%. Five isolates were identified as *N. hiratsukae* with a bootstrap value of 100%, four were identified as *N. pseudofischeri* (bootstrap value, 100%), two were identified as *A. viridinutans* (bootstrap value, 97%), two were identified as *A. fumigatiaffinis* (bootstrap value, 92%), and one was identified as *A. fumisynnematus* (bootstrap value, 98%). None of the *Aspergillus* section *Fumigati* sequences analyzed matched the *A. fumigatus* sequences.

Figure 2 shows the results of a phylogenetic analysis obtained by maximum parsimony of the rodlet A gene sequences. According to those results, the 28 clinical strains fell into the same six clades. Fourteen samples were grouped with *A. lentulus* with bootstrap values of 63% and 81%. Five isolates were identified as *N. hiratsukae* with a bootstrap of 100%. Four isolates were identified as *N. pseudofischeri*, two were identified as *A. viridinutans*, two were identified as *A. fumigatiaffinis*, and one was identified as *A. fumisynnematus*, supported by bootstrap values of 99%, 100%, 46%, and 83%, respectively. All *A. fumigatus* sequences were divided into a single group according to their rodlet A sequences.

The identities and classification results for the *Aspergillus* section *Fumigati* strains on the basis of their β-tubulin and rodlet A sequences are summarized in Table 1.

Antifungal susceptibility testing. The MICs and MECs of the antifungal agents for the collection of clinical isolates are shown in Table 1 and are expressed as geometric means (GMs). The species were identified by β-tubulin and rodlet A gene sequencing. Analyses of the susceptibility phenotypes (Table 1) resulted in clear differences between *Aspergillus* species of the section *Fumigati*. We were able to differentiate three different antifungal phenotypes. Twelve of the 14 *A. lentulus* isolates and the two *A. fumigatiaffinis* isolates showed high MICs of amphotericin B (GM MICs, 4.5 μg/ml to 16 μg/ml), itraconazole (GM MICs, 6 μg/ml to 10.25 μg/ml), voriconazole (GM MICs, 3 μg/ml to 7.5 μg/ml), and ravuconazole (GM

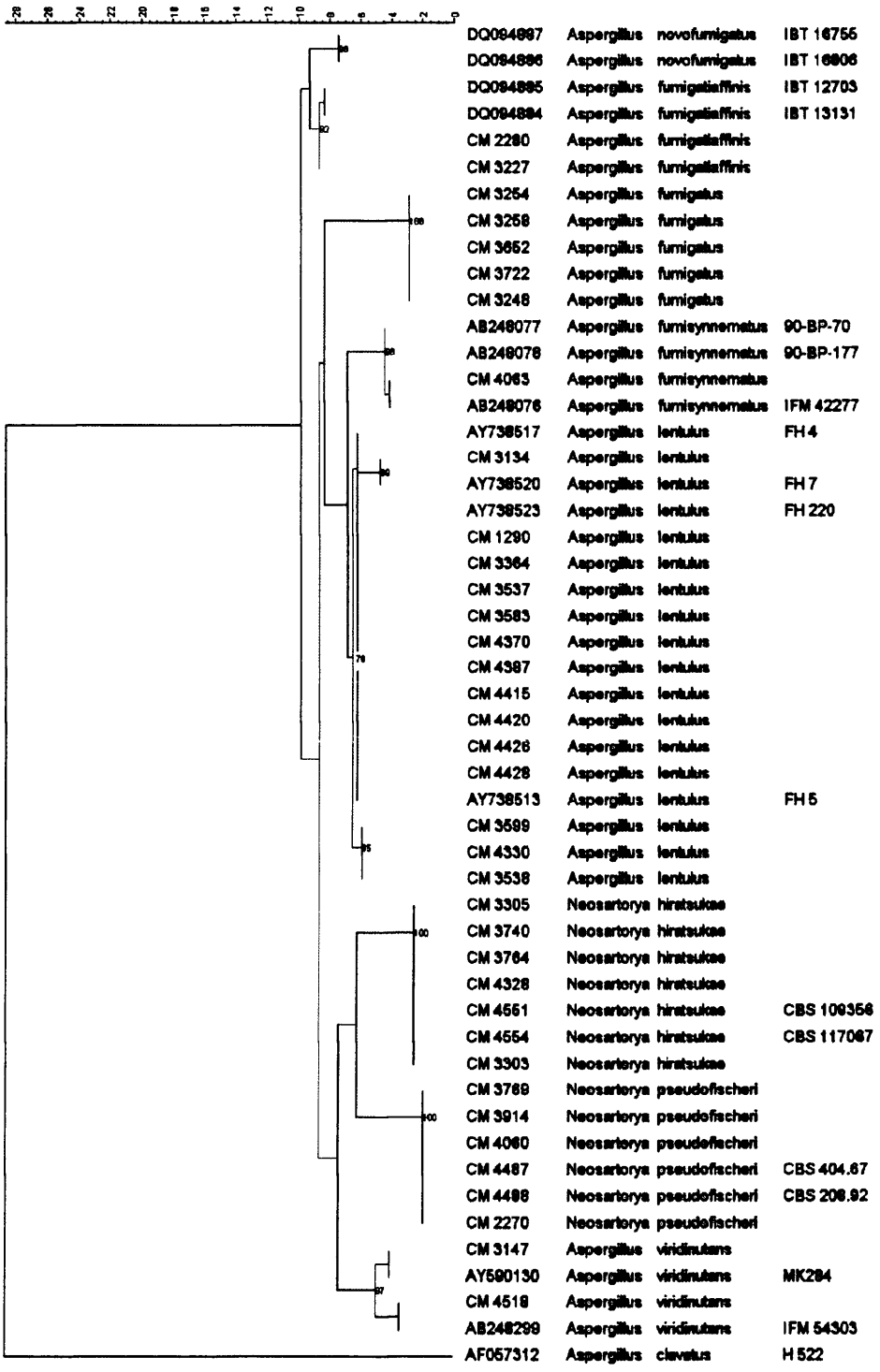

FIG. 1. Phylogenetic tree obtained by maximum-parsimony phylogenetic analysis with 2,000 bootstrap simulations on the basis of the β-tubulin sequences from all the strains included in the study.

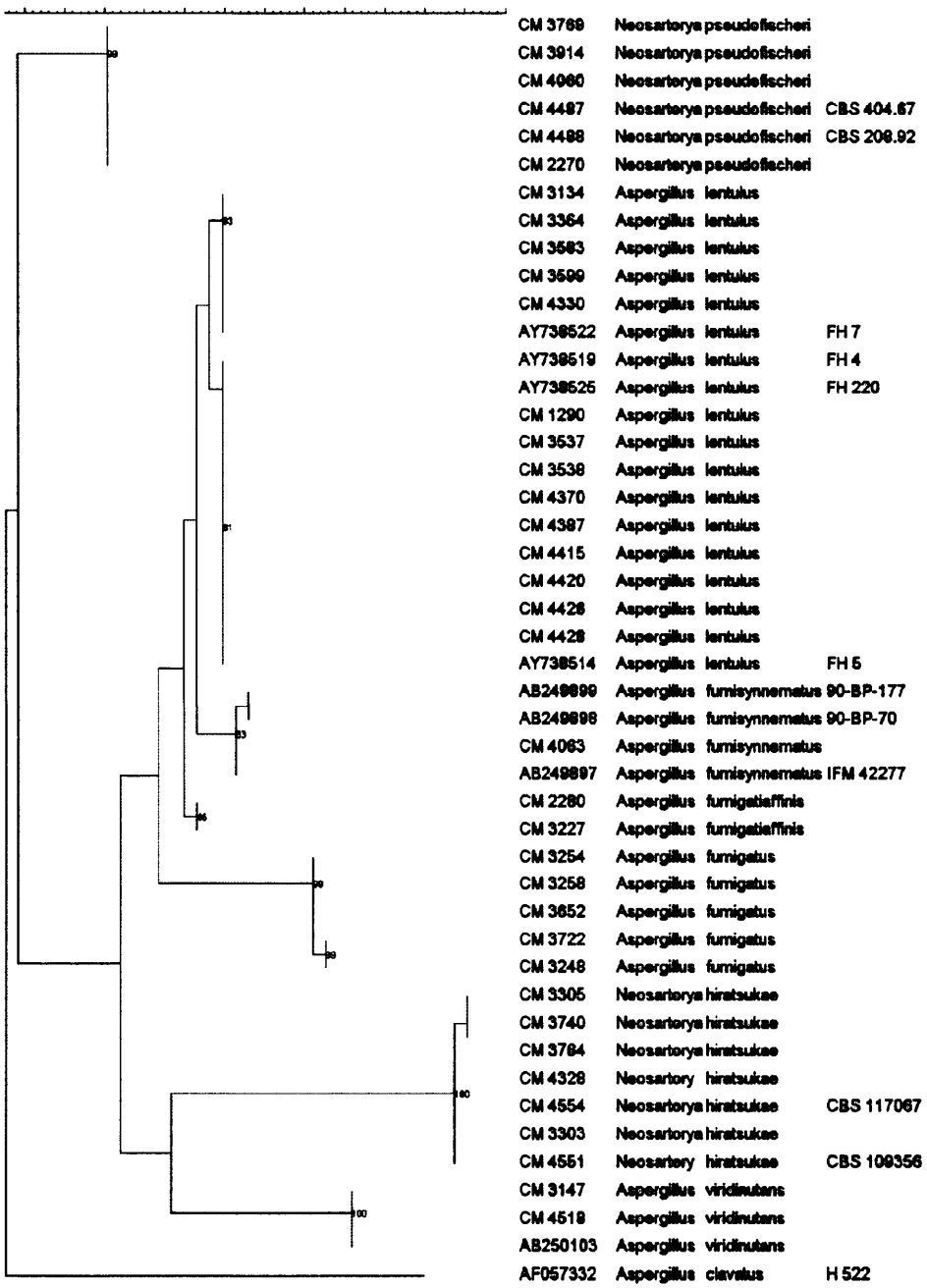

FIG. 2. Phylogenetic tree obtained by maximum-parsimony phylogenetic analysis with 2,000 bootstrap simulations on the basis of the rodlet A sequences from all the strains included in the study.

MICs, 1.5 μg/ml to 7 μg/ml). Although strains CNM-CM-3583 and CNM-CM-3599 did not follow this pattern for itraconazole (GM MICs, 0.43 μg/ml and 0.5 μg/ml, respectively), they retained the high MICs of amphotericin B (GM MICs, ≥2.66 μg/ml), voriconazole (GM MICs, ≥3.5 μg/ml), and ravuconazole (GM MICs, 2 μg/ml). A second profile was related to the species *N. pseudofischeri* and *A. viridinutans*. They showed high MICs of itraconazole (GM MICs, ≥8 μg/ml), voriconazole (GM MICs, ≥3.33 μg/ml), and ravuconazole (GM MICs, ≥2 μg/ml) but were susceptible to amphotericin B (GM MICs, ≤1

μg/ml). In contrast, strains identified as *N. hiratsukae* and *A. fumisynnematus* were more susceptible in vitro to all the antifungal compounds tested.

Among the azoles, posaconazole showed better activity in vitro (GM MICs, ≤0.75 μg/ml) against all clinical isolates analyzed in this study. Moreover, all strains were susceptible to terbinafine (GM MICs, ≤1.75 μg/ml) and the echinocandins, showing GM MECs of caspofungin and micafungin of ≤1.3 μg/ml and ≤0.26 μg/ml, respectively.

DISCUSSION

The present study highlights the limitations of phenotypic methods for the identification of some genera of molds. The use of molecular methods to partially sequence the β-tubulin and rodlet A genes enabled us to identify to the species level all clinical strains included in the study and previously classified as "atypical" *A. fumigatus* isolates. The majority of molecular methods use either specific probes or universal primers that are normally directed to conserved regions of the rRNA gene, particularly to the internal transcribed spacer regions (32, 35). However, internal transcribed spacer regions do not have enough phylogenetic strength to resolve the evolutionary relationship with strong bootstrap support for *Aspergillus* species from the section *Fumigati* (4, 5, 24, 41). Sequence analysis of both the β-tubulin and the rodlet A genes revealed that this method accurately differentiated the non-*A. fumigatus* isolates from the *A. fumigatus* isolates (4, 5). Therefore, all clinical isolates used in this study were identified to the species level by maximum-parsimony analysis of the β-tubulin and the rodlet A gene sequences.

On the basis of the identities of β-tubulin and rodlet A DNA sequences, the 28 strains were divided into six monophyletic clades supported by bootstrap values that differentiated the species (Fig. 1 and 2). The clades identified the isolates as belonging to the following species: 14 strains were *A. lentulus*, 5 strains were *N. hiratsukae*, 4 strains were *N. pseudofischeri*, 2 strains were *A. viridinutans*, 2 strains were *A. fumigatiaffinis*, and 1 strain was *A. fumisynnematus*.

A. lentulus has been isolated from soil and air, and it has also been isolated from patients with invasive infections (4). In this regard, most *A. lentulus* isolates recovered in this study were cultured from respiratory samples. This fact demonstrates the potential invasiveness of this pathogen in a susceptible host because *A. fumigatus* is able to colonize the human respiratory tract. *A. viridinutans*, *N. hiratsukae*, and *N. pseudofischeri* have also been isolated from humans (44), although only *N. hiratsukae* and *N. pseudofischeri* have been associated with human invasive fungal infections (3, 14, 17).

A. fumisynnematus was described as a separate taxon on the basis of the partial cytochrome *b* gene sequences of the species (43). However, this species was not included in the last classification of *Aspergillus* section *Fumigati* because the type strain of the species was not available for phenotypic characterization (36). To our knowledge, *A. fumisynnematus* and *A. fumigatiaffinis* had always been isolated from environmental sources. We describe for the first time the isolation of *A. fumisynnematus* and *A. fumigatiaffinis* strains from human hosts, which raises the intriguing issue of whether these species should be considered pathogenic fungi.

The common antifungal susceptibility phenotype of *A. fumigatus* is characterized by low MICs of the azole drugs, amphotericin B, and echinocandins. *A. fumigatus* strains showing resistance to azole drugs have been reported, although they have always maintained low amphotericin B MICs (11, 25, 26). In contrast, most *A. lentulus* and *A. fumigatiaffinis* isolates analyzed here showed extremely high MICs of amphotericin B, itraconazole, voriconazole, and ravuconazole.

Although 2 of the 14 *A. lentulus* isolates seemed to be susceptible to itraconazole, it is important to emphasize that we have not observed a uniform itraconazole susceptibility pattern, even after repeating the susceptibility testing more than eight times (data not shown). Due to the slow growth of most of these species, endpoint reading was easier at 72 h than at 48 h.

Since it has been demonstrated that elevated MICs of amphotericin B are associated with poor clinical outcomes (19, 30), the high MICs of amphotericin B for *A. lentulus* and *A. fumigatiaffinis* could have a remarkable clinical impact that merits research. In addition, it is well known that *A. terreus* strains have higher amphotericin B MICs than *A. fumigatus* strains, and this fact has been associated with a poorer response to amphotericin B in patients infected with this species (21, 37).

Even though *A. fumisynnematus* seems to be very closely related to *A. lentulus*, it had a different profile of susceptibility to all drugs tested. However, more isolates need to be analyzed in order to establish the antifungal susceptibility profile for this species.

Consistent with previous data (3, 44), the antifungal phenotypes of *N. pseudofischeri* and *A. viridinutans* showed that they had high itraconazole, voriconazole, and ravuconazole MICs.

All *N. hiratsukae* isolates were susceptible to all the antifungal drugs tested. There has been only one report of cerebral aspergillosis caused by *N. hiratsukae* (14), in which the strain cultured from that patient showed a pattern of MICs similar to the patterns described in this study.

Among the azoles, posaconazole showed the highest level of activity against all species analyzed. Terbinafine also had good activity in vitro against all clinical isolates compared with the MICs for *A. fumigatus*.

Finally, our antifungal susceptibility testing results for the echinocandins showed that all strains were susceptible to both caspofungin and micafungin (MECs, ≤1.3 μg/ml). This result contradicts the findings of other authors, but since a reference method for testing the susceptibilities of filamentous fungi to echinocandins has not yet been defined, conclusions about the echinocandin susceptibility or resistance of *Aspergillus* spp. should be made carefully.

This study emphasizes that molecular methods are needed for the correct identification of members of *Aspergillus* section *Fumigati* to the species level. Moreover, the members of this section have different antifungal susceptibility profiles. The identification of species or isolates showing high MICs of the antifungals used clinically is therefore mandatory. It should be noted that high in vitro MICs may not necessarily reflect decreased susceptibility in vivo, especially because these species have growth differences that could affect the results. Therefore, to ascertain the true clinical importance of these species, epidemiological studies must be performed together with in

vitro-in vivo correlation studies. In the meantime, it is difficult to give practical advice for clinical laboratories, but we suggest that those isolates which appear to be *A. fumigatus* under the microscope but which have poor sporulation or slow growth be sent to reference laboratories. An alternative approach could be to send any *A. fumigatus*-like strain which does not grow at 48°C to a reference laboratory. Due to the shortage of data and the unpredictable susceptibility profiles of some isolates, we highly recommend that antifungal susceptibility testing be performed by a standardized methodology for all isolates associated with human infections.

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3.1.3. *Aspergillus* sección *Nigri*

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Species Identification and Antifungal Susceptibility Patterns of Species Belonging to *Aspergillus* Section *Nigri*[▽]

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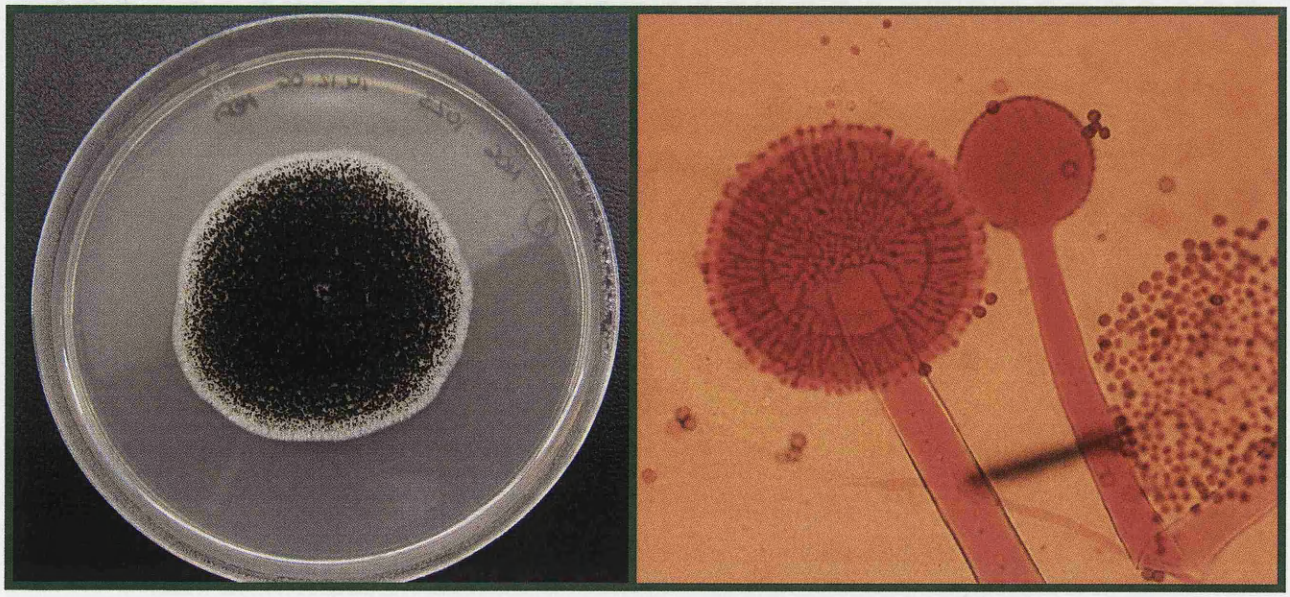
Resumen: Se analizaron filogenéticamente 34 cepas de *Aspergillus* pertenecientes a la sección *Nigri*. Los métodos moleculares permitieron la correcta clasificación de las cepas en tres especies (*Aspergillus niger, Aspergillus tubingensis* y *Aspergillus foetidus).* Se diferenciaron tres perfiles de sensibilidad in vitro al itraconazol: susceptible, resistente y aquellos que mostraron un efecto paradójico.



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A phylogenetic analysis was performed for 34 *Aspergillus* strains belonging to section *Nigri*. Molecular methods allowed for the correct classification into three different clades (*A. niger*, *A. tubingensis*, and *A. foetidus*). Correlation with in vitro itraconazole susceptibility distinguished the following three profiles: susceptible, resistant, and showing a paradoxical effect. A number of different species whose morphological features resemble those of *A. niger* showed unusual MICs to itraconazole that have never been described for the *Aspergillus* genus.

Black aspergilli are widely distributed in nature (16); they are common food spoilers but are also well used for industrial purposes (15). Among *Aspergillus* species of the *Nigri* group, *A. niger* constitutes the most frequent etiological agent of otomycosis (13) and is considered the third cause of pulmonary aspergillosis (10). Nevertheless, the clinical implications of other species are rarely reported, and they are generally identified as *A. niger* (14, 22).

Clinically, identification of unknown *Aspergillus* clinical isolates to the species level may be important given that different species have dissimilar susceptibilities to antifungal drugs. Thus, the knowledge of the species identity may influence the choice of appropriate antifungal therapy (2, 4). Furthermore, since the antifungal susceptibility patterns for most of the species within section *Nigri* have been poorly investigated, their identification and antifungal susceptibility profiles appear to be of clinical interest for further research.

Black aspergilli belong to one of the most difficult groups concerning classification and identification (18), and so a number of different techniques have been developed in order to solve this issue. Among them, molecular tools are the gold standard (1, 18), as the sequencing of the β-tubulin or calmodulin gene is suitable, and enough, to discriminate between species within section *Nigri* (3, 18, 21).

Thirty-four *Aspergillus* section *Nigri* strains belonging to the Mold Collection of the Centro Nacional de Microbiologia and collected since 2004 were analyzed. Thirty-three strains were independent clinical isolates, and 1 had an environmental origin. All strains were identified as *A. niger* using conventional methods of morphology at the macroscopic as well as microscopic levels (9). Species identification analysis was addressed using sequences of the β-tubulin gene from all the strains included in this study together with the sequences of different *Aspergillus* section *Nigri* type strains and others that were available at GenBank as follows: *A. tubingensis* AY820007^T, AY820009, and AY585527; *A. foetidus* AY585533^T, AY585534, and DQ768454; and *A. niger* FJ629288^T, EF422213, and AY585537. Partial sequences of the β-tubulin gene were amplified using the primer set βtubAniger1 and βtubANiger2 (11) and were carried out according to standard PCR guidelines (Applied Biosystems). Sequences were assembled and edited using the SeqMan II and EditSeq software packages (Lasergene 8.0; DNAStar, Inc., Madison, WI).

All phylogenetic analyses were conducted with InfoQuest FP software, version 4.50 (Bio-Rad). The methodology used was maximum parsimony clustering. Phylogram stability was assessed by using parsimony bootstrapping with 2,000 simulations and by using the *Aspergillus clavatus* AY214441^T sequence as the out-group.

The phylogenetic tree grouped the 34 clinical isolates into three different clades consisting of 13 *A. niger* isolates, 18 *A. tubingensis* isolates, and 3 *A. foetidus* isolates (Fig. 1). Table 1 shows β-tubulin gene identification, as well as the origin and susceptibility profiles of the isolates.

Antifungal susceptibility testing (AST) was performed following the EUCAST Definitive Document E.DEF 9.1 method for the determination of broth dilution MICs of antifungal agents for conidium-forming molds (17). Antifungal ranges used in the microdilution assays have been described previously (2). Endpoints were determined at 48 h. The endpoint for MEC determination was the minimal antifungal concentration that produced morphological alterations of hyphal growth at 48 h. The paradoxical effect to itraconazole was defined as an increase in growth occurring at least 2 drug dilutions above the MIC. AST was repeated at least twice on different days.

Three different antifungal patterns were clearly distinguishable based on the itraconazole MIC values (Table 1): low and high itraconazole MICs and a third group (12 strains) showing an uncommon paradoxical effect of this antifungal (5). Either those strains classified as paradoxical strains or those showing much higher itraconazole MICs also had higher MIC values to voriconazole and ravuconazole.

Posaconazole showed better activity in vitro. Moreover, all

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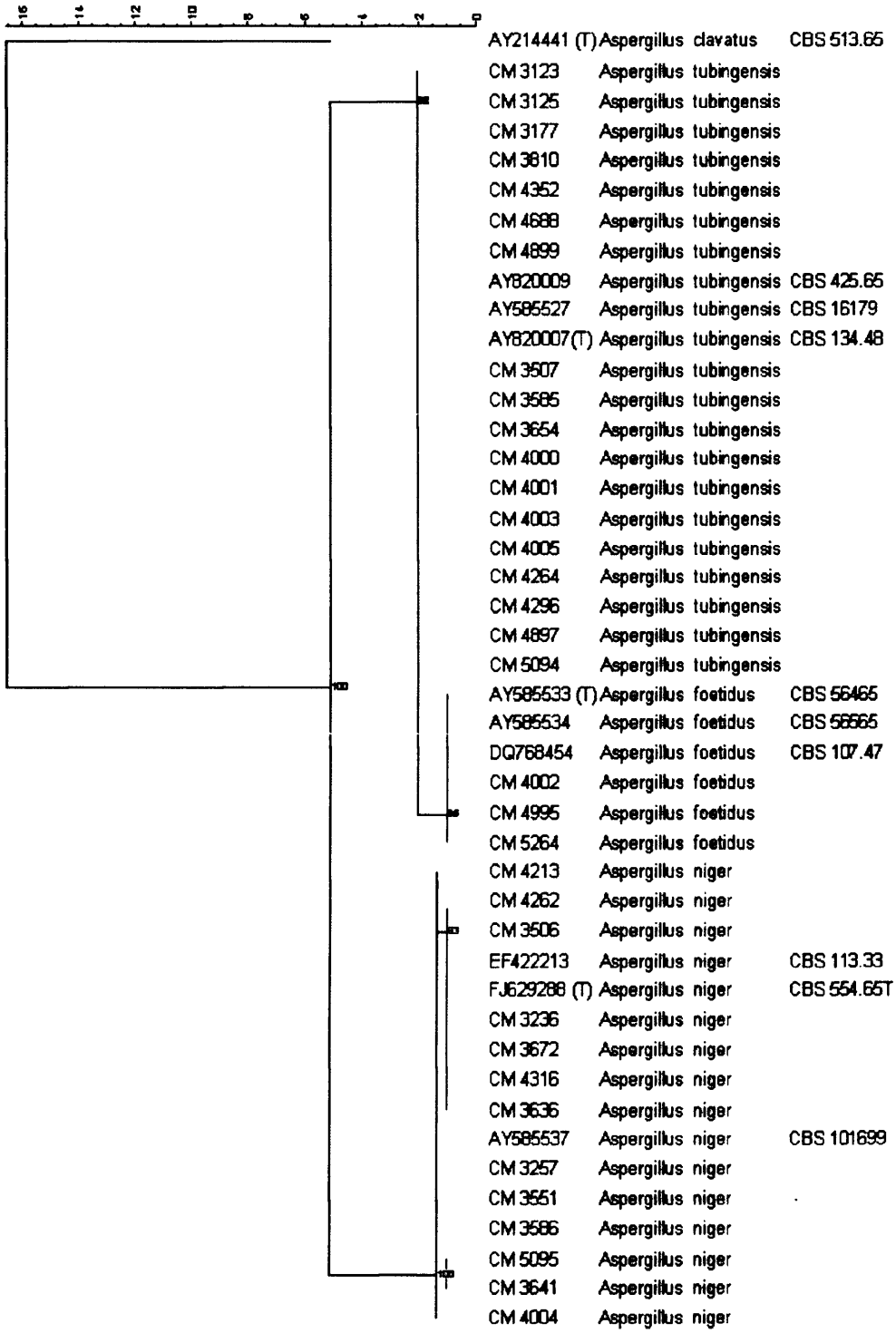

FIG. 1. Phylogenetic tree using maximum parsimony phylogenetic analysis and 2,000 bootstrap simulations based on β-tubulin gene sequences from all the *Aspergillus* section *Nigri* strains included in the study. Percentages indicate the bootstrap support for each group of sequences. (T), type strain.

TABLE 1. Source, molecular identification, MICs, and MECs for species of *Aspergillus* section *Nigri*[a]

Isolate	Source	Molecular identification (β-tubulin gene)	MIC (mg/liter)[b]						MEC (mg/liter)[c]	
			AMB	ITC	VCZ	RVC	POS	TRB	CAS	MICA
Isolates of *Aspergillus* section *Nigri* showing low ITC MICs										
CM-3236	Respiratory	*A. niger*	0.19	0.5	0.5	1.0	0.12	1.0	0.25	0.03
CM-3257	Respiratory	*A. niger*	0.25	1.0	1.0	1.0.	0.25	1.0	0.25	0.03
CM-3506	Respiratory	*A. niger*	0.19	0.5	0.75	1.0	0.12	0.31	0.15	0.03
CM-3507	Respiratory	*A. tubingensis*	0.19	0.5	1.0	1.5	0.15	0.62	0.06	0.03
CM-3585	Environmental	*A. tubingensis*	0.19	0.5	1.0	1.67	0.12	0.42	0.37	0.03
CM-3586	Catheter	*A. niger*	0.25	0.5	1.0	2.0	0.12	0.12	1.0	0.03
CM-3636	Respiratory	*A. niger*	0.25	0.5	0.5	1.0	0.19	1.0	0.25	0.03
CM-3641	Respiratory	*A. niger*	0.25	0.5	1.0	1.0	0.125	0.25	0.5	0.03
CM-3672	Cutaneous	*A. niger*	0.12	0.5	1.0	1.5	0.19	0.07	0.15	0.03
CM-4004	Unknown	*A. niger*	0.25	1.0	1.0	1.67	0.25	0.13	0.10	0.03
CM-4213	Respiratory	*A. niger*	0.33	0.14	0.33	0.58	0.03	0.22	0.39	0.03
CM-4264	Blood culture	*A. tubingensis*	0.12	0.5	1.0	1.5	0.12	0.50	0.03	0.03
CM-4296	Respiratory	*A. tubingensis*	0.12	0.75	1.0	1.5	0.19	0.62	0.25	0.03
CM-4316	Respiratory	*A. niger*	0.19	0.5	0.5	1.0	0.125	0.5	0.25	0.03
CM-5094	Respiratory	*A. tubingensis*	0.12	0.5	0.75	2.0	0.06	0.62	0.19	0.03
CM-5095	Respiratory	*A. niger*	0.19	0.5	0.75	1.5	0.12	0.62	0.25	0.03
GM for group			0.20	0.56	0.82	1.31	0.14	0.50	0.28	0.03
Isolates of *Aspergillus* section *Nigri* showing much higher ITC MICs										
CM-3123	Respiratory	*A. tubingensis*	0.25	11	1.67	2.67	0.25	1.17	0.25	0.03
CM-3810	Respiratory	*A. tubingensis*	0.25	4.0	2.0	2.0	0.12	1.0	0.5	0.03
CM-4003	Unknown	*A. tubingensis*	0.12	16	2.0	4.0	0.25	1.0	0.25	0.03
CM-4005	Unknown	*A. tubingensis*	0.12	16	2.0	4.0	0.5	0.25	0.5	0.03
CM-4688	Respiratory	*A. tubingensis*	0.21	3.67	2.0	3.33	0.25	1.50	0.18	0.03
CM-5264	Respiratory	*A. foetidus*	0.12	16	2.0	8.0	0.5	0.5	0.06	0.03
GM for group			0.18	11.11	1.95	4.0	0.31	0.90	0.29	0.03
Isolates of *Aspergillus* section *Nigri* showing paradoxical effect against ITC										
CM-3125	Respiratory	*A. tubingensis*	0.12	0.5	1	1.67	0.12	0.5	0.05	0.03
CM-3177	Respiratory	*A. tubingensis*	0.16	1	2	3.33	0.25	0.67	0.05	0.03
CM-3551	Respiratory	*A. niger*	0.5	4.75	1	2	0.12	0.25	0.03	0.03
CM-3654	Blood culture	*A. tubingensis*	0.19	1	2	2.50	0.25	0.63	0.14	0.03
CM-4000	Unknown	*A. tubingensis*	0.16	1	2	2	0.25	0.33	0.10	0.03
CM-4001	Unknown	*A. tubingensis*	0.19	1	1.75	2.50	0.25	0.56	0.11	0.03
CM-4002	Unknown	*A. foetidus*	0.25	1	2	2.67	0.12	0.33	0.14	0.03
CM-4262	Ophthalmic	*A. niger*	0.25	1	2	2	0.25	0.29	0.13	0.03
CM-4352	Respiratory	*A. tubingensis*	0.28	1	0.88	2	0.25	0.31	0.15	0.03
CM-4897	Blood culture	*A. tubingensis*	0.16	1	2	2	0.25	0.42	0.10	0.03
CM-4899	Respiratory	*A. tubingensis*	0.16	1	2	2.67	0.25	0.33	0.10	0.05
CM-4995	Prosthesis	*A. foetidus*	0.21	1	2	2	0.16	0.33	0.14	0.03
GM for group			0.2	1.3	1.72	2.28	0.21	0.41	0.10	0.03

[a] GM, geometric means of MICs and MECs for the strains within each group.
[b] MIC geometric mean of amphotericin B (AMB), itraconazole (ITC), voriconazole (VCZ), ravuconazole (RVC), posaconazole (POS), and terbinafine (TRB).
[c] MEC geometric mean of caspofungin (CAS) and micafungin (MICA).

strains were susceptible to the rest of the following antifungals tested: amphotericin B, terbinafine, and echinocandins.

In summary, *A. niger* MICs for itraconazole, voriconazole, and ravuconazole were slightly higher than *A. fumigatus* MICs and even more so for *A. tubingensis* and *A. foetidus* MICs. Identification of clinical isolates belonging to *Aspergillus* section *Nigri* and involved in proven or probable infections should be to the species level because it is the only way to monitor the development of secondary resistances of these molds (7, 8).

The paradoxical effect or "Eagle effect" (12) has been previously described for yeasts or *A. fumigatus* but always in relation to echinocandins (5, 6, 19, 20). This is the first report

showing the paradoxical effect of azole drugs against *Aspergillus* spp. The link between the paradoxical effect against itraconazole and a molecular mechanism responsible for it is yet to be determined, as is the clinical impact of those findings. Therefore, further studies including experimental models of aspergillosis to address any in vitro/in vivo correlations are warranted.

Nucleotide sequence accession numbers. GenBank accession numbers for β-tubulin gene fragment sequences from all the strains used in this work are as follows: CM-3123:FJ828892, CM-3125:FJ828893, CM-3177:FJ828894, CM-3236:FJ828895, CM-3257:FJ828896, CM-3506:FJ828897, CM-3507:FJ828898, CM-3551:FJ828899, CM-3585:FJ828900, CM-3586:FJ828901, CM-3636:FJ828902, CM-3641:FJ828903, CM-3654:FJ828904, CM-3672:FJ828905, CM-3810:FJ828906, CM-4000:FJ828907, CM-4001:FJ828908, CM-4002:FJ828909, CM-4003:FJ828910, CM-4004:FJ828911, CM-4005:FJ828912, CM-4213:FJ828913, CM-4262:FJ828914, CM-4264FJ828915, CM-4296:FJ828916, CM-4316:FJ828917, CM-4352:FJ828918, CM-4688:FJ828919, CM-4897:FJ828920, CM-4899:FJ828921, CM-4995:FJ828922, CM-5094:FJ828923, CM-5095:FJ82892, and CM-5264:FJ828925.

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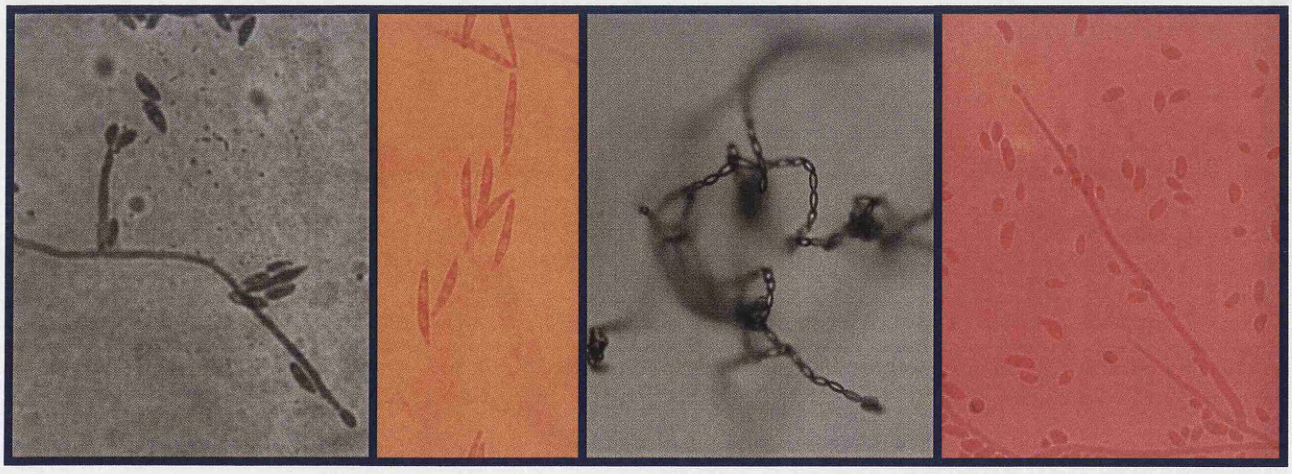
3.2. *Fusarium* spp.

3.2.1. Antifungal susceptibility profile of clinical *Fusarium* spp. isolates identified by molecular methods.

Journal of Antimicrobial Chemotherapy 2008; 61(4):805-809.

3.2.2. Disseminated fusariosis caused by *Fusarium verticillioides* in an acute lymphoblastic leukemia patient after allogeneic hematopoietic stem cell transplantation.

Journal of Clinical Microbiology 2009; 47(1):278-281.



3.2.1. *Fusarium* spp.

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Antifungal susceptibility profile of clinical *Fusarium* spp. isolates identified by molecular methods

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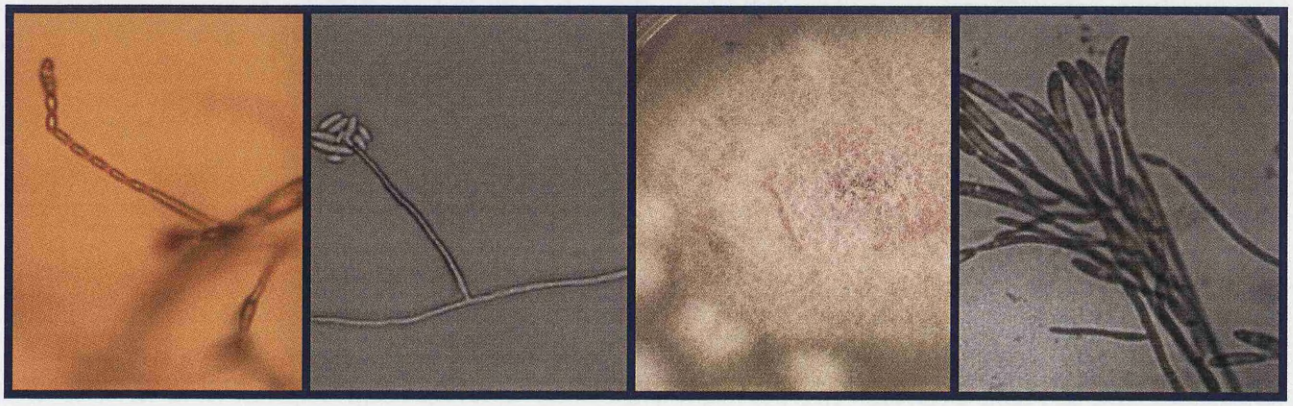
Resumen: Objetivos: Analizar el patrón de sensibilidad de una colección de aislados clínicos de *Fusarium.* Métodos: Se analizó el patrón de sensibilidad de 67 cepas de *Fusarium.* Las cepas fueron identificadas por métodos morfológicos y moleculares mediante la secuenciación del factor de elongación 1 alfa. Resultados y conclusiones: Se identificaron seis especies diferentes. La especie más frecuente fue *Fusarium solani* seguida de *Fusarium oxysporum, Fusarium proliferatum* y *Fusarium verticillioides.* La anfotericina B fue el fármaco más activo in vitro (intervalo: 0,015–32 mg/L). El resto de los antifúngicos ensayados (itraconazol, voriconazol, ravuconazol, posaconazol y terbinafina) mostraron poca actividad frente a las especies de *Fusarium* confirmando la naturaleza multiresistente de este género.



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***Objectives*: To analyse the susceptibility pattern of a collection of *Fusarium* clinical isolates.**

***Methods*: The antifungal susceptibility pattern of 67 isolates of *Fusarium* was analysed. Strains were identified by morphological and molecular methods by means of sequencing elongation factor α.**

***Results and conclusions*: Six different species were identified. *Fusarium solani* was the most frequently isolated, followed by *Fusarium oxysporum*, *Fusarium proliferatum* and *Fusarium verticillioides*. Amphotericin B was the only drug with *in vitro* activity (range: 0.015–32 mg/L). The rest of the antifungals tested (itraconazole, voriconazole, ravuconazole, posaconazole and terbinafine) showed very poor activity against *Fusarium*, confirming the multiresistant nature of this genus.**

Keywords: elongation factor α, antifungal resistance, emerging moulds

Introduction

Fusarium is a ubiquitous fungus widely distributed in soil, plants and different organic substrates. *Fusarium* species are important as plant pathogens causing different diseases and being responsible for important economic loss. During recent years, they have been increasingly associated with humans and now represent the second most frequent mould causing invasive fungal infections in immunosuppressed patients associated with high morbidity and mortality rates.[1,2]

The genus currently contains over 100 species. The most common pathogens are *Fusarium solani* and *Fusarium oxysporum* although other species have been reported as aetiological agents of human infection.[3–5] Identification to species level of *Fusarium* has been based on the study of their morphological characteristics. However, isolates involved in human infections usually do not produce the characteristic morphology that allows its identification.[6] Thus, recognition of *Fusarium* to species level is a laborious and time-consuming task only reserved to trained mycologists.[7] In order to solve this issue, molecular techniques have been developed to identify this genus.[8,9]

Fusarium spp. are resistant *in vitro* to many of the antifungal compounds licensed to treat fungal infections, and among them, *F. solani* is considered the most resistant. However, some data pointed out that the resistance could be species and even isolate dependent.[10] The management of fusariosis is not well defined. Antifungals alone or in combination together with other measures such as surgical intervention or colony stimulating growth factors have been used to treat these infections.[11] However, the mortality rate exceeds 75% in disseminated infections and an ominous outcome is expected without the recovery of the immunosuppression of the host.[12]

The susceptibility or resistance to antifungal agents may not predict the individual clinical outcome of *Fusarium* infections, but it is well-known that some kind of association between high MICs and poor response to antifungal treatment exists.[13,14] Therefore, the susceptibility profile of *Fusarium* spp. could be valuable as an aid to choose the best antifungal therapy. In addition, since susceptibility could be specific to one species, definitive identification at the species level by molecular methods may have clinical usefulness for the management of *Fusarium* infections.

The aim of this study is to analyse the activity *in vitro* of different antifungal compounds against a panel of clinical strains of *Fusarium* identified by a reference molecular technique,[9] consisting of partial sequencing of the translation elongation factor-1α (EF1α) gene.

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Materials and methods

Strains

A total of 67 clinical isolates of *Fusarium* spp. were included in this study. The isolates were obtained from a variety of clinical sources. Twenty-four strains were isolated from skin or nails, 16 from ocular samples, 13 from respiratory sites, 7 from blood cultures, 1 from urine, 1 from pericardial fluid and 5 of unknown origin. Each isolate was obtained from a different patient. The isolates were sent to the Mycology Reference Laboratory of National Centre for Microbiology of Spain during 2001–2007 for identification and susceptibility testing.

Morphological identification

The strains were subcultured in different media to ascertain their macroscopic and microscopic morphology. The media included malt extract agar (2% malt extract) (Oxoid S.A., Madrid, Spain), potato dextrose agar (Oxoid S.A.), oat meal agar (Oxoid S.A.) and potassium chloride agar (CIK, Oxoid S.A.).

All media were incubated at 30°C except for CIK agar, which was incubated at room temperature with cycles of 12 h of light followed by 12 h of dark.

PCR and DNA sequencing of EF1α region

Moulds were cultured in GYEP medium (0.3% yeast extract, 1% peptone, Difco, Soria Melguizo S.A., Madrid, Spain) with 2% glucose (Sigma-Aldrich Quimica, Madrid, Spain) for 24–48 h at 30°C. Genomic DNA was isolated using an extraction procedure described previously.[15]

DNA segments comprising a region of the EFα region were amplified with primers EF1 (5′-ATGGGTAAGARGACAAGAC-3′) and EF2 (5′-GGARGTACCAGTSATCATGTT-3′),[8] in a GeneAmp PCR System 9700 (Applied Biosystems). The reaction mixtures contained 0.5 μM of each primer, 0.2 μM of each deoxynucleoside triphosphate, 5 μL of PCR 10× buffer (Applied Biosystems, Madrid, Spain), 2.5 U of *Taq* DNA polymerase (Amplitaq; Applied Biosystems) and 25 ng of DNA in a final volume of 50 μL. The samples were amplified in a GeneAmp PCR System 9700 (Applied Biosystems) by using the following cycling parameters: one initial cycle of 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 45 s at 47°C and 2 min at 72°C, with one final cycle of 5 min at 72°C. The reaction products were analysed in a 0.8% agarose gel.

Sequencing reactions were done with 2 μL of a sequencing kit (BigDye Terminator cycle sequencing, ready reaction: Applied Biosystems), 1 μL of the primers (EF1 and EF2) and 3 μL of the PCR product in a final volume of 10 μL.

Sequences analysis

Sequences were assembled and edited using the SeqMan II and EditSeq software packages (Lasergene; DNAstar, Inc., Madison, WI, USA). Sequence analysis was performed by comparison of the DNA sequences with EFα sequences of *Fusarium* spp. strains (with accession nos: DQ246834, DQ247188, AY337433, AY337436, AF008480, AY337437, AJ543560, AJ543570, DQ295140, DQ295141, DQ295142 and DQ246834) obtained from the GenBank database (http://www.ncbi.nih.gov/GenBank/).

Phylogenetic analysis

All phylogenetic analyses were conducted with InfoQuest FP software, version 4.50 (BIORAD Laboratories, Madrid, Spain). The methodology used was maximum parsimony clustering. Phylogram stability was assessed by parsimony bootstrapping with 2000 simulations.

Antifungal susceptibility testing

Microdilution testing was performed following the CLSI reference method,[16] with the following minor modifications: (i) RPMI 1640 was supplemented with glucose to reach a 2% concentration; and (ii) inoculum size was between 1×10^5 and 5×10^5 cfu/mL. Inocula were prepared by means of counting spores in a haemocytometer.[17–19] *Aspergillus fumigatus* ATCC 2004305 and *Aspergillus flavus* ATCC 2004304 were used as quality control strains.[16]

The antifungal agents used in the study were amphotericin B (range 16–0.03 mg/L) (Sigma-Aldrich Química), itraconazole (range 8–0.015 mg/L) (Janssen S.A., Madrid, Spain), voriconazole (range 8-0.015 mg/L) (Pfizer S.A.), ravuconazole (range 8–0.015 mg/L) (Bristol-Myers Squibb, Princeton, NJ, USA), posaconazole (range 8–0.015 mg/L) (Schering-Plough Research Institute, Kenilworth, NJ, USA) and terbinafine (range 16–0.03 mg/L) (Novartis, Basel, Switzerland). The endpoint was the antifungal concentration that produced a complete inhibition of visual growth at 48 h.

Results

Identification of Fusarium to species level

All isolates were identified to genus level by means of observation of morphology characteristics.[6,20] The morphological identification of *F. solani*, *F. oxysporum* and *Fusarium verticilloides* is straightforward. However, the proper identification of other species such as *Fusarium proliferatum*, *Fusarium reticulatum* or *Fusarium equiseti* requires sequencing of EFα. In any case, the confirmation of species was obtained by means of maximum parsimony analysis of the EFα sequences. Figure 1 shows a rooted cladogram with a sample of clinical isolates. All clades in the tree had bootstrap values of 100 which support the use of EFα as correct target for molecular identification of *Fusarium* spp. In order to facilitate the visualization of the cladogram, some isolates were not included in Figure 1, but the species distribution and the bootstrap values were identical. Among 67 clinical strains, 22 were *F. solani*, 14 *F. oxysporum*, 14 *F. proliferatum*, 13 *F. verticilloides*, 3 *F. equiseti* and 1 *F. reticulatum*.

Antifungal susceptibility testing

The geometric means (GMs) and ranges of the MICs of antifungal agents are shown in Table 1. In all experiments performed, MICs for quality control strains were in the expected range.

Amphotericin B was the most active agent against *Fusarium* spp., its GM MIC being 1.15 mg/L. The numbers of isolates for which MICs of amphotericin B were ≥2 mg/L differed depending on the species: 12 out of 22 (54.6%) *F. solani*, 9 out of 14 (64.3%) *F. proliferatum*, 4 out of 13 (30.8%) *F. verticilloides* and 1 out of 14 (7.1%) *F. oxysporum* had MICs ≥2 mg/L.

Azole drugs and terbinafine had high MICs for most *Fusarium* spp. (Table 1). Only 12.1% of strains tested with posaconazole had MICs of ≤1 mg/L, dropping to 4.5% for itraconazole and voriconazole.

Regarding species, based on our *in vitro* data, the most resistant isolates in this study were *F. solani* for which GM MICs, of

Antifungal susceptibility profile of clinical *Fusarium* spp. isolates

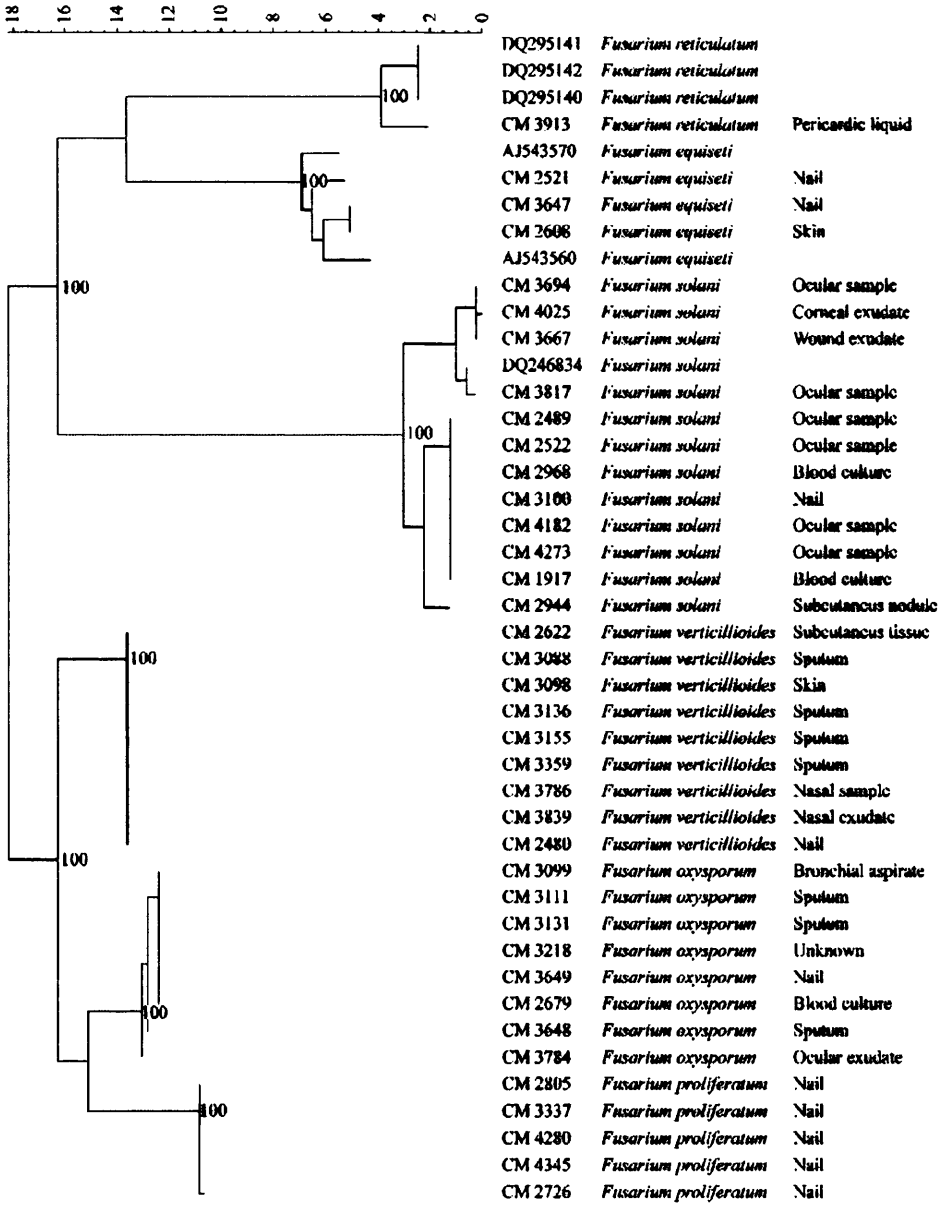

Figure 1. Phylogenetic tree of the subset of isolates included in the study obtained by using maximum parsimony phylogenetic analyses and 2000 bootstrap simulations based on EF1α sequences.

itraconazole, voriconazole, ravuconazole, posaconazole and terbinafine were >8 mg/L, but where the GM MIC of amphotericin B was 1.33 mg/L.

Discussion

Identification of moulds to species level by classical methods[6] is a cumbersome and time-consuming task. The expertise required is only available in reference laboratories, and even in them, at least five working days are required to identify a mould isolate to a species level by means of morphology observation. New rapid methods are needed in order to accomplish the identification on time to be useful for clinical management of the patient. Rapid molecular methods are being developed and they will probably replace the classical ones in the near future. In the meantime, proper identification of clinical isolates together with an antifungal susceptibility profile of them can help provide better treatment for patients infected with moulds. One of the main advantages of molecular methods is their sensitivity and

Table 1. Antifungal susceptibility results of clinical isolates of *Fusarium* spp.: MIC GMs and ranges

	MIC (mg/L)											
	amphotericin B		itraconazole		voriconazole		ravuconazole		posaconazole		terbinafine	
Fusarium spp.	GM	range	GM	range	GM	range	GM	range	GM	range	GM	range
F. solani (22)	1.33	0.5–8	16	16–16	14	4–16	16	16–16	16	16–16	29.96	16–32
F. verticilloides (13)	1.53	0.5–32	10.44	1–16	8	1–16	8.98	2–16	3.23	0.25–16	3.23	1–32
F. oxysporum (14)	0.78	0.12–2	11.31	1–16	4	0.5–16	8	1–16	4.63	0.06–16	10.77	0.5–32
F. proliferatum (14)	1.56	1–4	16	16–16	9.28	4–16	15.23	8–16	11.89	2–16	3.62	1–32
F. equiseti (3)	0.79	0.5–1	16	16–16	4	4–4	16	16–16	2	2–2	10.08	4–16
F. reticulatum (1)	0.015		16		1		1		1		0.25	
Total (67)	1.15	0.015–32	13.7	1–16	8	0.5–16	11.89	1–16	7.43	0.06–16	8.79	0.25–32

specificity, being fully discriminative even for closely related species. The majority of molecular methods are PCR-based techniques and use either specific probes or universal primers that are normally directed to conserved regions of the ribosomal DNA gene, particularly to the internal transcribed spacer (ITS) regions.[3,21,22]

In the case of *Fusarium* spp., sequencing of ITS analysis is considered unreliable for identification of strains because they contain two paralogous, discrepant ITS sequence types, which are a potential source of confusion.[23,24] Other genes have been used for the identification of *Fusarium* spp. and EFα has shown optimal results.[9,24,25] In this study, we have performed molecular identification with EFα and we have been able to differentiate among all the species analysed, as shown in Figure 1.

Up to now, over 15 species of *Fusarium* have been reported to cause infections in humans and animals, the most frequent aetiological agents being *F. solani* and *F. oxysporum*, but other species such as *F. verticilloides*, *Fusarium chlamydosporum*, *Fusarium dimerum*, *Fusarium napiforme*, *Fusarium nygamai*, *F. proliferatum* and *Fusarium sacchari* have also been reported in several cases of human infections.[5,23,26] In our collection, 46% of the strains belonged to species relatively infrequent as *F. proliferatum* (14 isolates), *F. verticilloides* (13), *F. equiseti* (3) and *F. reticulatum* (1). No other species encountered in other studies and related with human clinical samples were identified in this study. This fact could have been due to ecological reasons but it might be also due to not all fungi isolated for human sources being sent to a reference laboratory.

In this study, no activity *in vitro* of azoles drugs and terbinafine was detected against most of the isolates of *Fusarium* (Table 1). Amphotericin B has been the only drug that has shown activity *in vitro* against all the *Fusarium* species analysed with GM of 1.15 mg/L. Susceptible strains to this drug, with MICs of <2 mg/L were found: 13 out of 14 (92.9%) *F. oxysporum* isolates, 9 out of 13 (69.3%) *F. verticilloides*, 10 out of 22 (45.5%) *F. solani*, 5 out of 14 (35.7%) *F. proliferatum* and all isolates of *F. equiseti* and *F. reticulatum*. Azor *et al.*[27] have recently described the antifungal susceptibility profile of 50 clinical and environmental isolates of *F. solani*. MIC results were similar to those obtained in this work, amphotericin B being the most active drug.

Optimal treatment for *Fusarium* spp. has not yet been established. At best, response rates to antifungals such as lipid amphotericin B, voriconazole or posaconazole have ranged between 45% and 48%.[11,28,29] Kontoyiannis *et al.*[30] have analysed the impact on neutrophil recovery in the outcome of fusariosis. They concluded that this is the most important predictor of outcome. Other works have found similar results.[11,28,29] Taking into consideration that recovery from neutropenia is the most important factor, it would be better to treat these infections with the antifungal showing the highest *in vitro* activity against the isolate. In that way, we could gain enough time to enable the patient to recover a normal immune status. However, antifungal patterns regarding species could not be established by the data obtained and therefore we cannot make recommendations based on the identification of the isolate. However, as some isolates had lower MICs of amphotericin B, voriconazole and posaconazole, antifungal susceptibility testing could identify those isolates and help in the treatment of the patients.

Thus, as the susceptibility profile is isolate dependent, antifungal susceptibility testing should be performed for any *Fusarium* involved in an invasive fungal infection.

In summary, morphological and molecular identification of *Fusarium* species is cumbersome and should be restricted to laboratories with the required experience. An alternative for laboratories without the necessary experience could be the identification to genera level, but any strain isolated from a suspected invasive fungal infection should be identified to species level and its antifungal susceptibility profile determined. From a practical point of view, we have to bear in mind that *Fusarium* species is a multiresistant microorganism, as this work has demonstrated with a collection of clinical strains conclusively identified by molecular methods. However, as there are isolates with lower MICs, a joint effort should be initiated in order to determine if there is any kind of correlation among outcome of the patient, species identification and antifungal susceptibility profile of the isolate.

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Transparency declarations

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3.2.2. Caso clínico: *Fusarium verticillioides*

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Disseminated Fusariosis Caused by *Fusarium verticillioides* in an Acute Lymphoblastic Leukemia Patient after Allogeneic Hematopoietic Stem Cell Transplantation▽

Gulsum Tezcan,[1] Betil Ozhak-Baysan,[2] Ana Alastruey-Izquierdo,[3] Dilara Ogunc,[2]* Gozde Ongut,[2] Sinasi Taner Yıldıran,[4] Volkan Hazar,[1] Manuel Cuenca-Estrella,[3] and Juan Luis Rodriguez-Tudela[3]

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Resumen: Las especies de *Fusarium* son hongos filamentosos saprófitos que causan infecciones localizadas o diseminadas en humanos. Las infecciones diseminadas causadas por *Fusarium* llevan asociadas unas tasas de morbilidad y mortalidad en pacientes inmunodeprimidos muy altas. En este artículo se presenta el caso de un paciente con leucemia linfoblástica aguda con una fusariosis diseminada causada por *Fusarium verticillioides* y tratado satisfactoriamente con anfotericina B y voriconazol.

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***Fusarium* species are saprophytic molds which cause disseminated or localized infections in humans. Disseminated *Fusarium* infection can cause significant morbidity and mortality in immunocompromised patients. We present a case of disseminated fusariosis caused by *Fusarium verticillioides* in a patient with acute lymphoblastic leukemia and successfully treated using both liposomal amphotericin B and voriconazole.**

CASE REPORT

A 12-year-old boy underwent allogeneic hematopoietic stem cell transplant (allo-HSCT) from unrelated cord blood for high-risk acute lymphoblastic leukemia in its first complete remission. The conditioning regimen included busulfex (12.8 mg/kg of body weight), etoposide (40 mg/kg), and cyclophosphamide (120 mg/kg). For graft-versus-host-disease prophylaxis, cyclosporine A was added on day −1 but switched to mycophenolate mofetil due to severe allergic reaction. By the fourth day after the transplant, he was in severe neutropenia ($<0.1 \times 10^3$ neutrophils/μl) and became febrile, and antibiotic treatment (meropenem) was initiated for febrile neutropenia. Although the fever disappeared within few days of antibiotic onset, on day 23 posttransplant, he again became febrile, and treatment with liposomal amphotericin B (LAmB) was then started, with a dosage of 3 mg/kg/day. Cultures of separate blood samples obtained percutaneously and from a central venous catheter yielded coagulase-negative *Staphylococcus epidermidis*, and teicoplanin was added. On day 59 posttransplant, the patient developed multiple skin lesions, starting from the extremities and spreading to the face and trunk. The lesions had necrotic centers surrounded by spreading erythema (Fig. 1). A biopsy of the skin lesion showed the presence of histopathological symptoms consistent with a septate pathogenic mold, and blood cultures taken on the same day were positive for a *Fusarium* species.

The diagnosis of disseminated fusariosis was established, and LAmB was raised to a dose of 5 mg/kg/day. During the days following, the lesions worsened and treatment with voriconazole (loading dose, 6 mg/kg/day, followed by 4 mg/kg/day intravenously every 12 h) was initiated. A chest X-ray and a high-resolution computed tomography scan of the lungs were normal. Since profound neutropenia still continued, it was accepted that the patient had graft failure and new, unrelated donor search was started. Since a fully HLA-matched donor was not available, the patient underwent peripheral blood stem cell transplantation from a 9/10-matched, unrelated donor on day 82 posttransplant. The conditioning regimen included cyclophosphamide (120 mg/kg), and for graft-versus-host-disease prophylaxis, antithymocyte globulin (60 mg/kg), mycophenolate mofetil, and methotrexate were used. Neutrophil engraftment occurred on day 13 posttransplant. Combined-antifungal therapy and antifungal therapy with only voriconazole were continued until the ends of first and third months after peripheral blood stem cell transplantation, respectively. Two weeks after discontinuation of voriconazole therapy, the patient developed swelling and increased heat in the right knee joint and tenderness on movement, consistent with the diagnosis of arthritis. The patient was not neutropenic. On the days following, other joints also became affected. Examination of synovial fluid obtained from the right knee disclosed two or three granulocytes, and it was transudative. Culture yielded *Fusarium* spp., and combined-antifungal therapy with LAmB and voriconazole was restarted, and although the severity of the symptoms reduced, they did not disappear completely. Successive blood cultures within 15 days were found sterile. LAmB therapy was continued for 1 month, and at the end of 1 month, he was discharged home on oral voriconazole alone.

The macroscopic and microscopic morphologies of isolated *Fusarium* sp. were examined, and it was identified as *Fusarium verticillioides* on the basis of these characteristics. In short, it has a cottony colony with white aerial mycelium tinged with purple with a colorless reverse macroscopically (Fig. 2). In microscopic examination, it was found to have septate and hyaline hyphae; conidiophores arising laterally from hyphae in the aerial mycelium, sparsely branched; and abundant micro-

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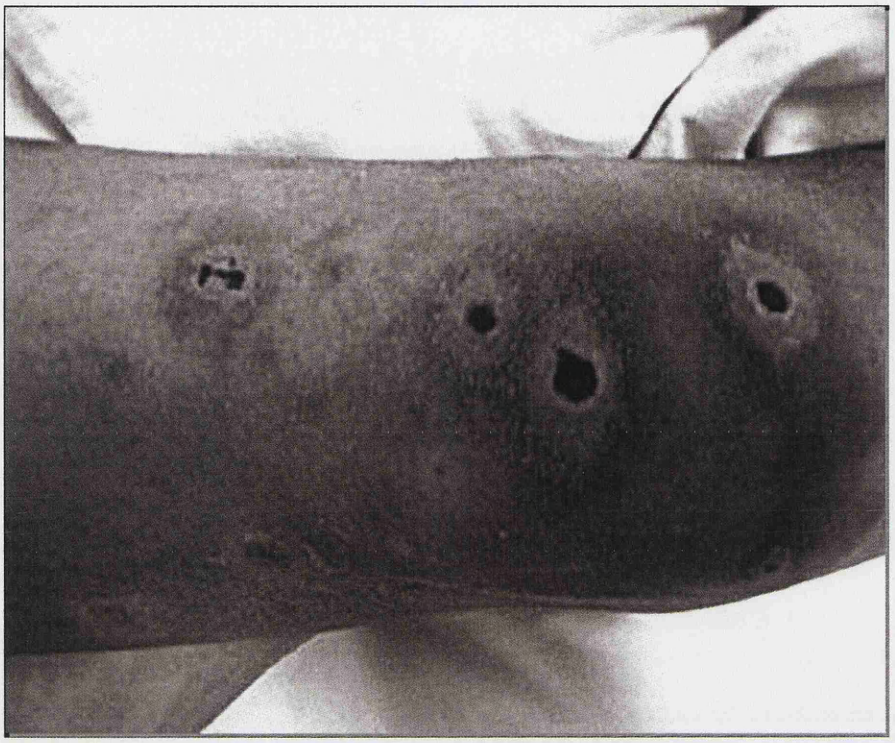

FIG. 1. Multiple necrotic skin lesions on the patient's leg.

conidia, in chains, ovoidal to clavate. Conidiogenous cells are monophialidic (Fig. 3).

Molecular identification was performed in order to confirm the identification to the species level. The *Fusarium* isolate was cultured in GYEP medium (0.3% yeast extract, 1% peptone; Difco, Soria Melguizo S.A., Madrid, Spain) with 2% glucose (Sigma Aldrich Química, Madrid, Spain) for 24 to 48 h at 30°C. Genomic DNA was extracted by using a previously described procedure (10). DNA segments comprising a region of elongation factor alpha (EFα) and the internal transcribed spacers (ITS) were amplified with primers EF1 (5′-ATGGGTA AGARGACAAGAC-3′), EF2 (5′-GGARGTACCAGTS ATCATGTT-3′), ITS1 (5′-TCCGTAGGTGAACCTGCG G-3′), and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) in a GeneAmp 9700 PCR system (Applied Biosystems, Madrid, Spain) (11, 21). The reaction mixtures contained 0.5 μM of each primer, 0.2 μM of each deoxynucleoside triphosphate, 5 μl of 10× PCR buffer (Applied Biosystems), 2.5 U *Taq* DNA polymerase (Amplitaq; Applied Biosystems), and 25 ng of DNA in a final volume of 50 μl. The samples were amplified in a GeneAmp 9700 PCR system (Applied Biosystems) by using the following cycling conditions: 1 initial cycle of 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 45 s at 47°C (EFα) or 56°C (ITS), and 2 min at 72°C, with 1 final cycle of 5 min at 72°C. The reaction products were analyzed with a 0.8% agarose gel.

Sequencing reactions were done with 2 μl of a mixture from a sequencing kit (BigDye Terminator cycle sequencing ready reaction; Applied Biosystems), 1 μl of the primers (EF1, EF2, ITS1, and ITS4), and 3 μl of the PCR product in a final volume of 10 μl.

Sequences were assembled and edited using the SeqMan II and EditSeq software packages (Lasergene; DNAstar, Inc., Madison, WI). Sequence analysis was performed by comparison of the DNA sequences with EFα sequences of *Fusarium* strains (with accession numbers DQ246834, DQ247188, AY337433, AY337436, AF008480, AY337437, AJ543560, AJ543570, DQ 295140, DQ295141, DQ295142, and DQ246834) obtained from the GenBank database (http://www.ncbi.nih.gov/Genbank/).

All phylogenetic analyses were conducted with InfoQuest FP software, version 4.50 (Bio-Rad Laboratories, Madrid, Spain). Blood and synovial fluid isolates were identical according to DNA sequence analysis.

The in vitro susceptibilities of *F. verticillioides* to antifungal drugs were determined using the broth dilution method, following the European Committee for Antimicrobial Susceptibility Testing procedures (19). This standard is similar to the one published by the CLSI (formerly NCCLS) reference method for broth dilution antifungal susceptibility testing of filamentous fungi, but with the following minor modifications: (i) RPMI 1640 was supplemented with glucose to reach a 2% concentration and (ii) the inoculum sizes were between 1.0×10^5 and 5.0×10^5 CFU/ml (1, 3, 16, 18). Inoculum preparations were performed by counting spores in a hematocytometer. *Aspergillus fumigatus* ATCC 2004305 and *Aspergillus flavus* ATCC 2004304 were used as quality control strains.

The antifungal agents used in the study were amphoteri-

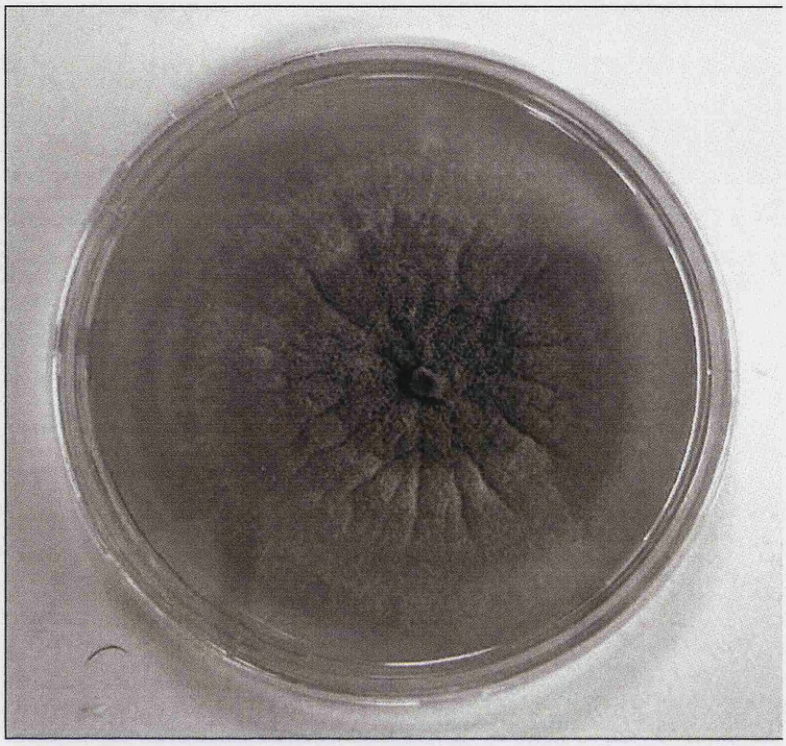

FIG. 2. Macroscopic colonies on Sabouraud dextrose agar with purple aerial mycelium.

cin B (range, 16 to 0.03 μg/ml) (Sigma Aldrich Química), itraconazole (range, 8 to 0.015 μg/ml) (Janssen Pharmaceutica S.A., Madrid, Spain), voriconazole (range, 8 to 0.015 μg/ml) (Pfizer S.A., Madrid, Spain), posaconazole (range, 8 to 0.015 μg/ml) (Schering-Plough Research Institute, Kenilworth, NJ), and terbinafine (range, 16 to 0.03 μg/ml) (Novartis, Basel, Switzerland). The endpoint was the antifungal concentration that produced a complete inhibition of visual growth at 48 h.

The antifungal susceptibility results for the blood isolate were as follows: amphotericin B, 1 μg/ml; itraconazole, >8 μg/ml; voriconazole, 4 μg/ml; posaconazole, >8 μg/ml; and terbinafine, 2 μg/ml.

The antifungal susceptibility results for the synovial fluid

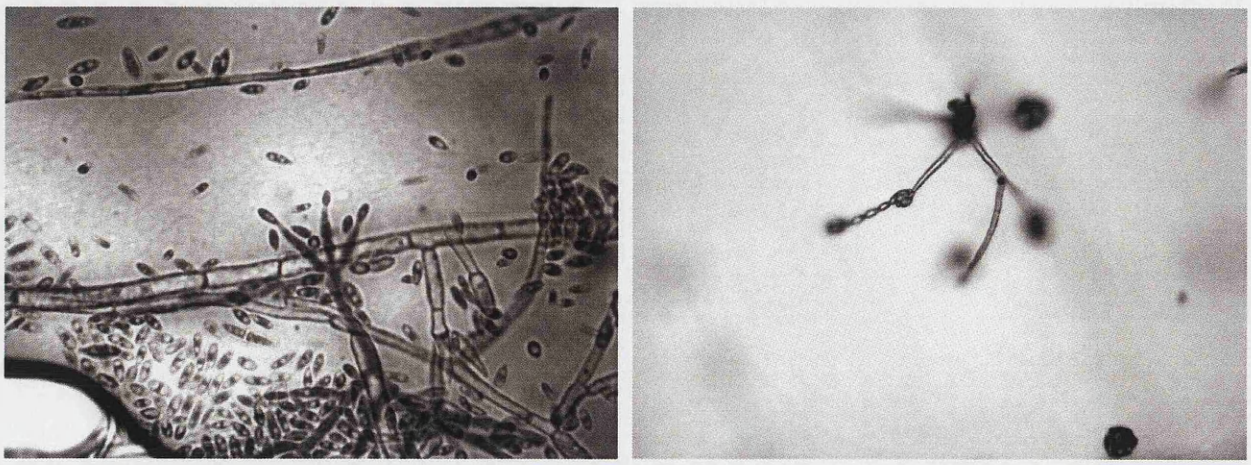

FIG. 3. (Left panel) Microscopy showing septate and hyaline hyphae, abundant microconidia, and monophialidic conidiogenous cells. (Right panel) Microscopic morphology of microconidia in chains in potassium chloride agar.

isolate were as follows: amphotericin B, 2 μg/ml; itraconazole, >8 μg/ml; voriconazole, 8 μg/ml; posaconazole, >8 μg/ml; and terbinafine, 4 μg/ml.

Fusarium species are saprophytic molds that are prevalent in the soil and air. They can cause local cutaneous infections and infections of surgical and burn wounds. However, this fungus has caught particular attention as an emerging pathogen of immunocompromised patients with involvement of multiple organs and numerous skin lesions (5, 13).

The *Fusarium* spp. most frequently involved in human infections are *Fusarium solani*, *Fusarium oxysporum*, and *Fusarium verticillioides* (6, 12). Of note, in tissue samples *Fusarium* spp. are often confused with *Aspergillus* spp., as both pathogens have similar histopathologic appearances, with septate, dichotomously branching hyphae (8). Yet, therapeutic options are scarce, and mortality reaches 80 to 90% in patients subjected to allo-HSCT (14, 20). We describe a pediatric allo-HSCT patient with disseminated fusariosis who responded to early combination of voriconazole and LAmB.

The epidemiology of fungal infections in hemopoietic stem cell transplant recipients has changed in the last 20 years. *Candida* species were still the most frequent agents of invasive mycoses (15). In recent years, invasive fungal infections caused by *Aspergillus* spp. and other emerging molds, such as *Fusarium* spp., have increased in frequency in hemopoietic stem cell transplant recipients (14). Invasive fusariosis can be life-threatening, and treatment options are limited. The increasing incidence, rapid progression, and high mortality rates of invasive fusariosis have necessitated early aggressive management of patients. There is no definitive effective treatment of invasive fusariosis. Currently, high-dose amphotericin B and its lipid formulations are the primary treatment for invasive fusariosis. Expanded-spectrum triazoles, particularly voriconazole and posaconazole, have also been used, and voriconazole is approved as front-line treatment for fusariosis (2, 15).

Fusarium spp. are very resistant to antifungal agents. Different species may exhibit variable susceptibility patterns. In the case of isolation of the organism, it is recommended that antifungal susceptibility testing be performed and that the active agent be administered at the highest tolerable dose (15). In the presented case, the MICs of amphotericin B and voriconazole were 1 μg/ml and 4 μg/ml, respectively. Because of the high mortality rate of invasive fusariosis under monotherapy, new treatment strategies, such as combination therapy, can be considered life-saving (9). At present, no randomized prospective studies evaluating combination therapy for invasive fusariosis are available. There are isolated case reports of successful treatment of invasive fusariosis with an amphotericin B and voriconazole combination. All of these case reports showed that the efficacy of combination therapy is better than that of monotherapy for fungal infections (4, 7, 17, 20). Our patient was successfully treated with combination therapy of amphotericin B and voriconazole, suggesting that the combination therapy is a potential alternative for patients with invasive fusariosis.

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3.3. Mucorales

3.3.1. Activity of posaconazole and other antifungal agents against Mucorales strains identified by sequencing of internal transcribed spacers.

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3.3.2. In vitro activity of antifungals against Zygomycetes

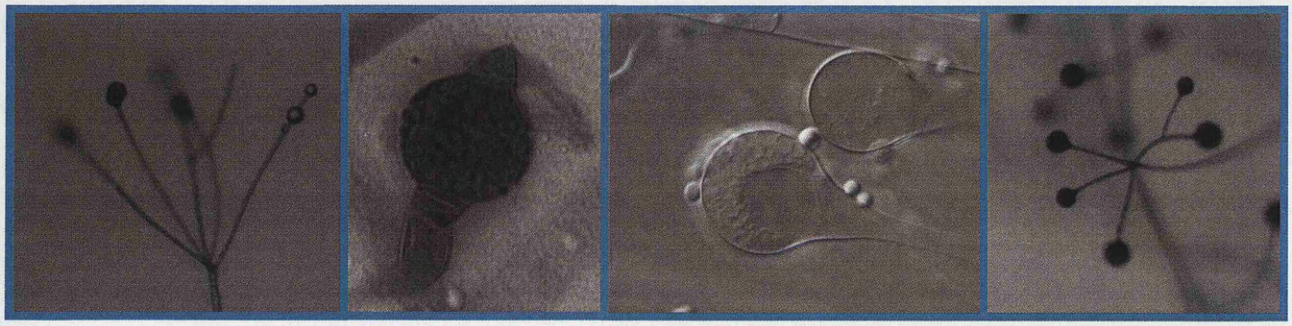
Clinical Microbiology and Infection, 2009 Oct;15 Suppl 5:71-6.

3.3.3. pecies recognition and clinical relevance of the zygomycetous genus *Lichtheimia* (syn. *Mycocladus*, *Absidia* pp.)

Enviado a publicar Journal of Clinical Microbiology

3.3.4. Antifungal susceptibility profile of human pathogenic species of *Lichtheimia*

Enviado a publicar Antimicrobial Agents and Chemotherapy



3.3.1. Actividad del posaconazol y otros antifúngicos frente a Mucorales

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Activity of Posaconazole and Other Antifungal Agents against *Mucorales* Strains Identified by Sequencing of Internal Transcribed Spacers▽

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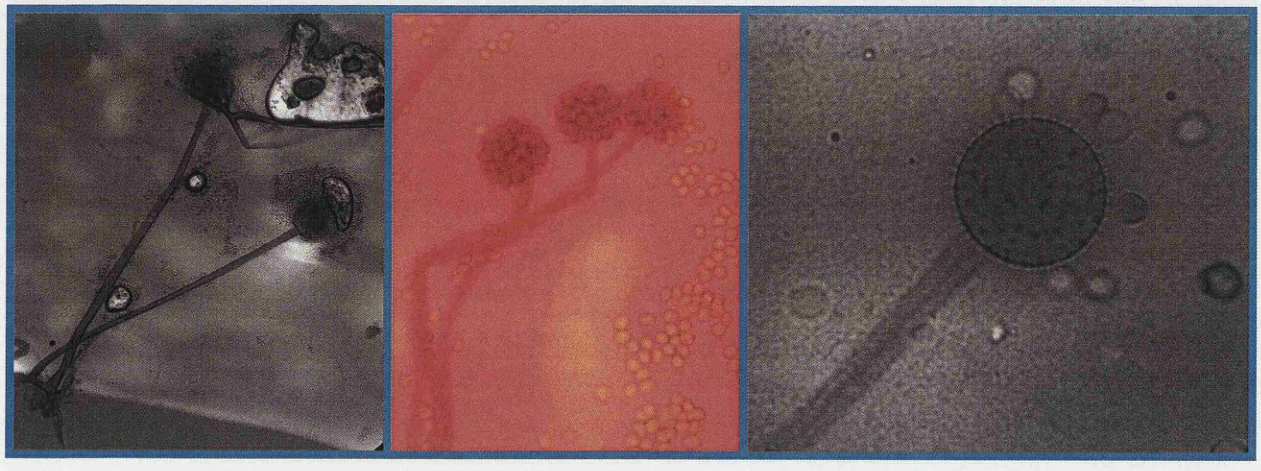
Resumen: Se analizó el perfil de sensibilidad a los antifúngicos de 77 aislados clínicos de mucorales identificados mediante la secuenciación de la región ITS. Se compararon los resultados de las CMIs obtenidas a las 24 y 48 horas. La Anfotericina B fue el antifúngico más activo frente a todas las especies menos *Cunninghamella* y *Apophysomyces*. El posaconazol mostró también buena actividad para todas las especies menos *Cunninghamella bertholletiae*. El voriconazol fue inactivo frente a todas las cepas ensayadas. La terbinafina tuvo buena actividad excepto para los aislados de *Rhizopus oryzae*, *Mucor circinelloides* y *Rhizomucor variabilis*.



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The antifungal susceptibility profiles of 77 clinical strains of *Mucorales* species, identified by internal transcribed spacer sequencing, were analyzed. MICs obtained at 24 and 48 h were compared. Amphotericin B was the most active agent against all isolates, except for *Cunninghamella* and *Apophysomyces* isolates. Posaconazole also showed good activity for all species but *Cunninghamella bertholletiae*. Voriconazole had no activity against any of the fungi tested. Terbinafine showed good activity, except for *Rhizopus oryzae*, *Mucor circinelloides*, and *Rhizomucor variabilis* isolates.

Mucormycosis (zygomycosis) is an aggressive and usually fatal human infection. The most common etiologic agents are *Rhizopus* spp., although other species have also been associated (19). Most cases of disease occur among immunocompromised individuals (18). Recently, an increase in the number of cases of mucormycosis has been observed (10, 11, 26). This increase has been particularly evident since the advent of voriconazole prophylaxis and treatment of aspergillosis infection in immunocompromised patients (20, 24, 27).

The gold standard therapy has not been well defined yet. It usually requires a combination of antifungal treatment, surgical intervention, and control of the underlying risk factors (19). The agent of choice for treating this infection is amphotericin B (5). However, mortality remains high, even with aggressive therapies. Posaconazole has been successfully used in salvage therapy for mucormycosis. In addition, other antifungals have potential utility in mucormycosis treatment.

Species identification is epidemiologically and clinically important, because *Mucorales* species can exhibit differences in their antifungal in vitro susceptibilities (4, 5). Unfortunately, identification by morphology examination requires a high level of expertise.

The in vitro activity of posaconazole and those of five other antifungals against 77 clinical *Mucorales* isolates identified by sequencing the internal transcribed spacer (ITS) ribosomal DNA region are compared. For *Mucorales* species, CLSI and the European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical Microbiology and Infectious Diseases recommend obtaining endpoint data at 24 h. However, since growth rates across the *Mucorales* species are not uniform, MICs obtained at 24 and 48 h were compared in order to ascertain whether there are differences in the results obtained at those two different times.

Identification of the strains. Seventy-seven clinical isolates of *Mucorales* species were obtained between 1999 and 2008 in the Mycology Reference Laboratory of the National Centre for Microbiology of Spain. All isolates were identified to the genus level by means of morphology determinations by following the usual procedures (7). In addition, all strains were identified to the species level by analyzing the ITS sequences by parsimony analysis as described before (2). *Rhizopus oryzae* and *Mucor circinelloides* were the species most frequently encountered. Smaller numbers of isolates of species of other genera such as *Rhizomucor*, *Cunninghamella*, and *Mycocladus* were found. Two strains of *Actinomucor elegans* and one of *Apophysomyces elegans* were also found.

The identification of members of the *Mucorales* genus to the species level is a hard task, even for well-trained mycologists. Kontoyiannis et al. (11) have shown discordance of more than 20% between ITS sequencing results and morphological identification for this group of fungi. Sequencing of appropriate targets should be considered the gold standard for identification. In this study, the analysis of the ITS sequences allowed us to identify all species, indicating that the ITS region is an appropriate molecular target for identification of these fungi. The only clade not supported was that formed by *Rhizomucor variabilis* and *M. circinelloides*. *R. variabilis* has previously been found to be more closely phylogenetically related to *Mucor* species (28). Molecular data, together with susceptibility profiling, also support this finding.

Antifungal susceptibility testing. Microdilution testing was performed by following the guidelines provided by the European Society of Clinical Microbiology and Infectious Diseases in European Committee for Antimicrobial Susceptibility Testing definitive document E.DEF 9.1 (1, 17, 22, 23). *Aspergillus fumigatus* ATCC 2004305 and *Aspergillus flavus* ATCC 2004304 were used as quality control strains (23). The antifungal agents used were amphotericin B (Sigma Aldrich Química S.A.), itraconazole (Janssen Pharmaceutica, Madrid, Spain), voriconazole (Pfizer S.A., Madrid, Spain), ravuconazole (Bristol-Myers Squibb, Princeton, NJ), posaconazole (Schering-Plough Research Institute, Kenilworth, NJ), and terbinafine (Novartis, Basel, Switzerland). Visual readings were performed with the help of a mirror at 24 and 48 h. The endpoint was the antifungal concentration that produced a complete

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TABLE 1. Geometric means and MIC ranges of the antifungal agents tested with the 77 *Mucorales* isolates

Organism (no. of isolates)	Antifungal agent	MIC (mg/liter) at 24 h		MIC (mg/liter) at 48 h	
		GM[a]	Range	GM	Range
Rhizopus oryzae (26)	Amphotericin B	0.29	0.03–32	1	0.125–32
	Itraconazole	4	0.125–16	6.96	0.25–16
	Voriconazole	12.13	4–64	16	8–64
	Ravuconazole	1	0.25–16	1.32	0.25–16
	Posaconazole	1.15	0.06–16	2	0.06–16
	Terbinafine	24.25	16–32	32	16–32
Mucor circinelloides (20)	Amphotericin B	0.05	0.015–0.125	0.15	0.06–1
	Itraconazole	11.89	1–16	16	1–16
	Voriconazole	16	8–64	16	1–64
	Ravuconazole	11.89	1–16	13.13	2–32
	Posaconazole	1.49	0.25–16	2.69	0.25–16
	Terbinafine	21.53	2–32	32	1–32
Mycocladus corymbifer (7)	Amphotericin B	0.08	0.03–0.25	0.14	0.06–0.25
	Itraconazole	0.68	0.125–16	1.35	0.25–16
	Voriconazole	14.49	8–16	16	16
	Ravuconazole	1.22	0.5–4	2	1–8
	Posaconazole	0.41	0.125–16	0.67	0.25–16
	Terbinafine	0.5	0.25–16	1	0.5–32
Cunninghamella bertholletiae (6)	Amphotericin B	7.13	2–32	7.13	2–32
	Itraconazole	3.17	0.5–8	10.08	4–16
	Voriconazole	16	8–32	16	8–32
	Ravuconazole	8	4–16	12.13	8–16
	Posaconazole	2.30	0.5–8	4.59	1–16
	Terbinafine	0.40	0.03–16	0.49	0.03–32
Rhizopus microsporus (6)	Amphotericin B	0.45	0.25–1	0.89	0.5–1
	Itraconazole	1.59	0.5–16	3.56	0.5–16
	Voriconazole	8	4–16	8.98	4–16
	Ravuconazole	0.89	0.25–16	1.78	0.25–8
	Posaconazole	0.79	0.25–8	3.17	0.5–16
	Terbinafine	0.71	0.125–32	1.41	0.25–32
Rhizomucor pusillus (5)	Amphotericin B	0.05	0.02–0.125	0.10	0.06–0.125
	Itraconazole	0.29	0.125–0.5	0.44	0.125–1
	Voriconazole	5.28	0.5–16	6.06	0.5–16
	Ravuconazole	0.66	0.25–2	0.87	0.25–2
	Posaconazole	0.16	0.06–0.25	0.28	0.06–0.5
	Terbinafine	0.22	0.125–0.5	0.33	0.25–0.5
Rhizomucor variabilis (2)	Amphotericin B	0.03	0.02–0.06	0.09	0.06–0.125
	Itraconazole	16	16	16	16
	Voriconazole	16	16	16	16
	Ravuconazole	8	4–16	16	16
	Posaconazole	1.41	1–2	16	16
	Terbinafine	32	32	32	32
Cunninghamella spp. (2)	Amphotericin B	2.83	2–4	4	4
	Itraconazole	0.5	NA[b]	4	1–16
	Voriconazole	16	16	16	16–16
	Ravuconazole	1	NA	8	4–16
	Posaconazole	0.25	NA	4	1–16
	Terbinafine	0.13	NA	0.18	0.125–0.25
Actinomucor spp. (2)	Amphotericin B	1	1	1.41	1–2
	Itraconazole	2	0.5–8	2	0.5–8
	Voriconazole	16	8–32	22.63	16–32
	Ravuconazole	0.25	0.25	0.5	0.5
	Posaconazole	0.06	0.06	0.125	0.125
	Terbinafine	0.125	0.06–0.25	0.36	0.125–1
Apophysomyces spp. (1)	Amphotericin B	2	NA	8	NA
	Itraconazole	16	NA	16	NA
	Voriconazole	16	NA	16	NA
	Ravuconazole	2	NA	4	NA
	Posaconazole	0.5	NA	1	NA
	Terbinafine	2	NA	2	NA

[a] GM, geometric mean.
[b] NA, not applicable.

TABLE 2. Isolates with >2-fold dilution differences in drug MICs between 24 and 48 h of incubation

Drug and species	No. of isolates	MIC (mg/liter) at 24 h	MIC (mg/liter) at 48 h
Amphotericin B			
Mucor circinelloides	2	0.015	0.125
	1	0.03	0.5
	1	0.03	0.25
Rhizopus oryzae	1	0.06	1
	1	0.06	0.5
	1	0.25	2
Itraconazole			
Cunninghamella spp.	2	0.5	16
Mucor circinelloides	1	2	16
Rhizopus oryzae	2	1	8
	1	1	16
	1	0.25	16
Rhizopus microsporus	1	1	8
Ravuconazole			
Cunninghamella spp.	1	1	16
Rhizopus oryzae	1	0.25	4
	1	0.5	4
Rhizopus microsporus	1	0.5	8
	1	1	8
Posaconazole			
Cunninghamella spp.	1	0.25	16
Mucor circinelloides	1	2	16
Rhizomucor variabilis	2	2	16
	1	1	16
Rhizopus microsporus	1	1	8
	1	0.5	16
Terbinafine			
Mucor circinelloides	2	2	32
	1	2	16

inhibition of visual growth (Table 1). As the growth rate of most *Mucorales* species is high, antifungal susceptibility reference methods (14, 23) recommend obtaining endpoint data at 24 h. For *Aspergillus fumigatus*, however, it has been proven that obtaining data at 48 h is mandatory in order to detect resistant strains (21). In this work we have detected several strains showing more-than-twofold differences in dilutions between endpoint data obtained at 24 h and those obtained at 48 h (Table 2). Although the relevance of this change in MICs is not known, it must be taken into consideration that all strains analyzed in this work were fully resistant to voriconazole at 24 h, supporting an earlier reading at 24 h as appropriate. However, although we still recommend reporting endpoints for *Mucorales* species at 24 h, further research performed with strains susceptible at 24 h and resistant at 48 h is needed.

Amphotericin B, which is the antifungal of choice for this mycosis, was the most active agent against all isolates, with the exception of those belonging to the genera *Cunninghamella* and *Apophysomyces*. High drug MICs for Cunninghamella species have been reported before (8, 9, 15, 29, 30). Terbinafine was active against all species tested, except for *R. oryzae*, *M. circinelloides*, and *R. variabilis*. Azole drugs showed various levels of activity. Itraconazole showed activity against only *R. pusillus* and *Mycocladus corymbifer*. Similar results have been found in other studies (5, 25). Itraconazole has also shown good activity in animal models of infection with *M. corymbifer* (6, 12). Therefore, itraconazole could be useful for some cases of mucormycosis when susceptible strains are involved. Voriconazole has no in vitro activity against these fungi. In addition, it has been shown that patients with leukemia and bone marrow transplant recipients on voriconazole prophylaxis can develop breakthrough infections caused by *Mucorales* species (11). Ravuconazole showed some activity against *M. corymbifer*, *R. pusillus*, *R. oryzae*, *R. microsporus*, and *Actinomucor elegans*, although isolates resistant to this drug were found in most of the species. Posaconazole was the azole drug which showed the best in vitro activity. The geometric mean of the MICs was ≤2 mg/liter for all species but *Cunninghamella bertholletiae*. This is the first drug in the azole class to show a broad spectrum of activity against the *Mucorales* species and has proven to be useful in combination with amphotericin B to treat rhinocerebral mucormycosis or patients for whom treatment with amphotericin B alone has failed (13, 16).

In summary, *Mucorales* species represent a heterogeneous group of fungi with various levels of susceptibility to antifungals (3) that can be easily identified by sequencing the ITSs of the ribosomal DNA. These differences in susceptibility occur among the same genera and species; therefore, a correct determination of the susceptibility profile for an individual clinical isolate is essential for devising the best therapy for mucormycosis.

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3.3.2. Actividad in vitro de los antifúngicos frente a Zygomycetes

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In vitro activity of antifungals against Zygomycetes

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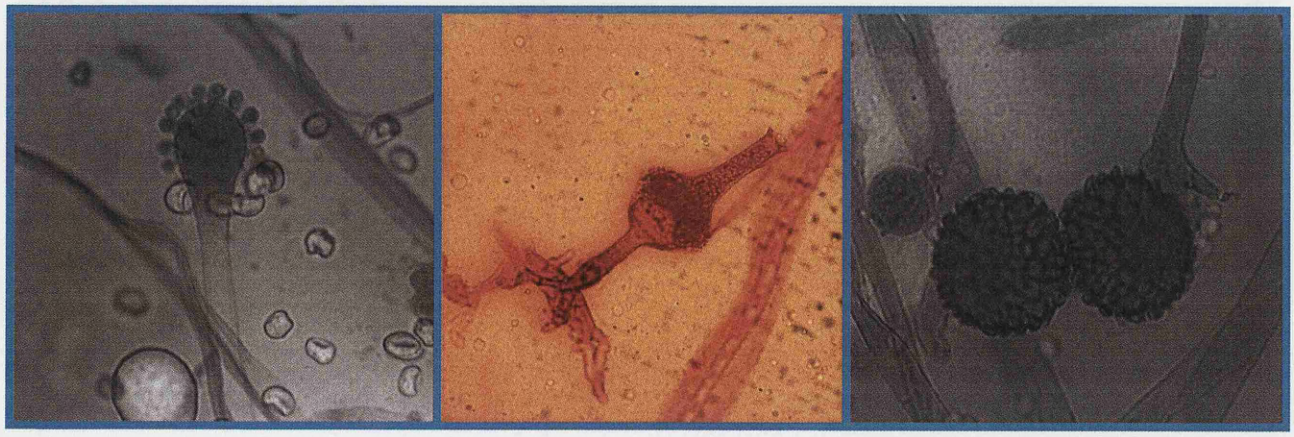
Resumen: La falta de datos clínicos hace que, por el momento, se desconozca el tratamiento de elección de la zygomycosis. La identificación del agente etiológico y el estudio de su perfil de sensibilidad son esenciales en el manejo de estas infecciones ya que la actividad de los antifúngicos es dependiente de cepa. La anfotericina B es el antifúngico más activo frente a todas las especies, seguida del posaconazol, mientras que el voriconazol carece de actividad. Las equinocandinas son inactivas in vitro, pero pueden suponer una alternativa en terapia combinada.



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Abstract

To date, no reference standard for therapy for zygomycosis has been established because there are insufficient clinical data with which to make such a judgement. Knowledge of the species responsible for the infection and its antifungal susceptibility profile has become increasingly important in the management of patients. Amphotericin B is the most active drug against all the species involved, followed by posaconazole, whereas voriconazole has no activity. Echinocandins are completely inactive *in vitro*, but may be an interesting option when used in combination with other drugs.

Keywords: Antifungal, in vitro activity, mucormycosis, Zygomycetes

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Introduction

In the last few years, the number of cases of zygomycosis has increased, especially among immunocompromised patients, although several authors have also reported infections in patients with unknown underlying conditions [1–3]. The course of the infection is rapidly progressive and potentially fatal, with high rates of mortality and morbidity. No reference standard for therapy has yet been established. Therapy usually requires a combination of measures, including antifungal treatment, surgical intervention and control of the underlying risk factors [4]. The agent of choice for treating this infection is amphotericin B (AmB) [5]. However, therapy with this drug has produced variable results; toxicity often occurs and the immune status of the patient plays an important role in the outcome, both of which highlight the importance of developing new strategies for treatment. Posaconazole has been used as salvage therapy for zygomycosis and has improved outcome. [6,7]. In addition, echinocandins have been used in combination therapies, underlining the potential utility of other antifungals in the treatment of zygomycosis. The low rates of response to these various therapies can be attributed to a range of factors, but knowledge of the species responsible for the infection and its antifungal susceptibility profile is of increasing value in the management of patients.

Unfortunately, identification by morphology examination of macroscopic and microscopic characteristics requires a high level of expertise. Kontoyiannis *et al.* [8] reported a 20% discrepancy between identification by means of morphology and that achieved by sequencing internal transcribed spacers. In addition, antifungal susceptibility testing data are limited and are based on isolates identified by their morphological characteristics [5,9,10].

The aim of this article is to review the antifungal susceptibility profile of the Zygomycetes in order to provide information for the better management and treatment of the life-threatening infections they cause.

Available Methodologies for Antifungal Susceptibility Testing in Zygomycetes

Two standardized methods are available for determining the susceptibility of moulds to antifungal agents. One method is the CLSI standard 'Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi' (Approved Standard M38-A) [11]. This document recommends the use of: (i) standard RPMI-1640 broth; (ii) non-germinated conidial inoculum suspensions of 10^4 CFU/mL, and (iii) for *Rhizopus* spp., incubation at 35 °C for 24 h. The subcommittee on Antifungal Susceptibility Testing (AFST) of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) has developed an alternative standard for

conidia-forming moulds [12]. The differences with the CLSI methods are: (i) RPMI-1640 is supplemented with glucose to reach a 2% concentration, and (ii) inoculum size is between 1×10^5 and 5×10^5 CFU/mL. Inoculum preparations are performed by means of counting spores in a haematocytometer [13–15]. Concordance between these two methods was studied by Chryssanthou and Cuenca-Estrella [16], who found a level of agreement of 92.5%.

Antifungal Susceptibility Profile of Zygomycetes

Table 1 shows a literature review of the antifungal susceptibility profile of Zygomycetes.

Amphotericin B

Of the antifungal treatments available, AmB shows the best *in vitro* activity against most of the species responsible for zygomycosis (Table 1) [9,17,18]. Unfortunately, these species have a broad range of susceptibilities to this drug [19,20]. *Cunninghamella* spp. and *Rhizopus* spp. have higher minimum inhibitory concentrations (MICs) to AmB, whereas *Mucor* spp. and *Absidia* spp. are subject to greater activity on the part of the drug (Table 1). The highest number of clinical failures relate to infections caused by *Cunninghamella bertholletiae*, which supports the high AmB MICs reported for this Zygomycete [2,21–25]. However, several series and case reports describing successful treatment with this drug have been published [26–37]. A number of retrospective studies have reported an increase in survival rates when lipid formulations of AmB were used as first-line or salvage therapy, primarily liposomal AmB (L-AmB) [26–33]. As these formulations are more effective and better tolerated, they have replaced conventional AmB in the treatment of these infections.

Azole drugs

Azole drugs have a limited *in vitro* activity against Zygomycetes. However, *in vivo* studies with animal models have shown that they can be active against zygomycosis [38,39]. In addition, posaconazole has been used as salvage therapy with positive results, constituting a promising alternative for the treatment of these infections.

Itraconazole

Although many authors have stated that itraconazole is not a good choice for treatment of zygomycosis, some cases of infection have been successfully treated with this drug [40–42]. *In vitro* results show that itraconazole is more active against Zygomycetes than voriconazole and that some strains are inhibited by low concentrations of itraconazole [18,43–45]. *In vitro* studies with itraconazole have shown a wide range of MICs (Table 1) [5,18,46]. Singh *et al.* [46] determined the itraconazole MICs for 15 strains of Zygomycetes, finding that *Rhizomucor*, *Syncephalastrum* and *Mycocladus* (*Absidia*) showed lower MICs of itraconazole (ranges 0.03–2 mg/L), whereas *Cunninghamella* and *Mucor* were more resistant (ranges 0.5 mg/L to >8 mg/L). These data are in accordance with findings reported by Dannaoui *et al.* [5], where *Mycocladus* and *Rhizomucor* were the two genera that showed lower itraconazole MICs. In addition, in a murine model of *Mycocladus corymbifer* infection, itraconazole therapy increased the survival rate of infected animals [47,48]. Therefore, itraconazole may be useful in some cases of zygomycosis in which susceptible strains are involved.

Voriconazole

Voriconazole is not active against Zygomycetes *in vitro*. All studies have shown MICs >2 mg/L. In most studies, MICs >8 mg/L have been reported [5,9,10,18,21,46]. In addition, it has been shown that patients with leukaemia or bone marrow transplantation recipients undergoing voriconazole prophylaxis can develop breakthrough infections caused by Zygomycetes [8, 49, 50].

Posaconazole

Posaconazole is the first drug in the azole drug family to show a broad spectrum of activity against Zygomycetes. *In vitro* studies have shown good activity against these fungi ($MIC_{50} \leq 1$ mg/L) [5,9,10,18]. The species which have shown higher MICs for this drug are *Rhizopus* spp. and *Cokeromyces recurvatus*, with a geometric mean of >2 mg/L, whereas *Absidia* spp. and *M. corymbifer* are the most susceptible species; *Saksenaea vasiformis* and *Rhizomucor* spp. also exhibit low MICs for posaconazole, although few strains have been tested (Table 1). In addition, experimental models of infection have proven the *in vivo* activity of this drug. Among mice treated with posaconazole, a survival increase occurred in mice infected with *Mucor* spp. [51], partial efficacy was seen in those infected with *M. corymbifer*, and a dose-dependent response was found in those infected with *Rhizopus microsporus* [52]. In addition, similar results have been obtained with posaconazole and AmB used as prophylaxis in neutropenic mice [53].

Two clinical studies have evaluated the efficacy of posaconazole as salvage therapy for zygomycosis. Van Burik *et al.* [7] reported a 60% response in 91 patients and Sun *et al.* [51] found a 79% response in 24 patients.

TABLE 1. ***In vitro* data of antifungal susceptibility of Zygomycetes to amphotericin B, itraconazole and posaconazole**

			Amphotericin B mg/L				Itraconazole mg/L				Posaconazole mg/L			
Species	*n*	References	Range	MIC_{50}	MIC_{90}	GM	Range	MIC_{50}	MIC_{90}	GM	Range	MIC_{50}	MIC_{90}	GM
Rhizopus spp.	15	[5]	0.06–1	0.5	1	0.42	0.25–32	0.5	4	0.87	0.125–1	0.25	0.50	0.27
	101	[9]	0.03–0.5	0.25	0.5		0.03 to >8	0.5	4		0.06–4	0.25	1	
	6	[57]	1–2	1			0.5–2	1						
	10	[18]	0.06–2	0.125	0.5	0.33	0.25–8	1	8	3.93	0.25–8	1	8	2.73
R. microsporus	12	[9]	0.03–0.5	0.25	0.25		0.25–1	0.5			0.25–2	0.25		
	1	[9]	0.25				1							
	1	[61]	0.25				>16							
	5	[21]	1–2	1	2		8 to >8	>8	>8					
R. oryzae	14	[21]	0.25–8	1	4		0.5 to >8	>128	>128					
	2	[46]	0.03–0.06			0.04	0.25–4			1.41				
	20	[9]	0.06–0.5	0.25	0.25		0.25–2	0.5			0.03–1	0.25	1	
		[43]	0.12–0.25	0.25	0.25		0.5–4	1	2					
	15	[10]		0.5	2			>8	>8			0.5	8	
Mucor spp.	6	[5]	0.03–0.25	0.125		0.09	1–32	8		6.96	0.5–2	1		1.15
	41	[9]	0.125–4	0.25	0.5		0.25 to >8	0.5	>8		0.06–2	0.5	2	
	6	[18]	0.06–0.5	0.25	0.25	0.24	0.25–8	1	2	2.18	0.125–8	0.5	1	1.54
M. circinelloides	6	[9]	0.06–0.5	0.25			2 to >8				1–2			
	1	[21]	0.25				8							
	3	[46]	0.03			0.03	0.5 to >8			2.82				
M. ramosissimus	3	[21]	0.12–0.5	0.25	0.5		1–8	2	8					
Absidia spp.	3	[9]	0.25–0.5				0.5–1				0.125			
	10	[5]	0.06–0.125	0.125	0.125	0.09	0.03–0.125	0.06	0.25	0.08	0.06–0.25	0.06	0.125	0.09
Mycocladus corymbifer	9	[9]	0.25–0.5	0.25			0.125–0.5				0.06–0.25			
	5	[18]	0.25–0.5	0.25	0.25	0.30	0.03–0.25	0.06	0.25	0.14	0.03–0.25	0.03	0.25	0.13
	4	[21]	0.25 to >16	0.50	>16		1 to >8	2	>8					
	3	[46]	0.03–0.25			0.05	0.125–2			0.62				
	1	[61]	0.06				0.03							
		[43]	0.06–0.25	0.25	0.25		0.25–0.5	0.25	0.5					
Rhizomucor spp.	3	[5]				0.06				0.09				0.09
	5	[9]	0.125–0.25	0.125			0.125–1				0.06–1			
R. pusillus	3	[46]	0.125–0.25			0.16	0.03–0.125			0.07				
Cunninghamella spp.	13	[9]	0.25–2	1			0.125–4	1			0.06–1	0.5		
	5	[18]	0.125–2	0.25	0.25	0.55	0.125–2	0.25	0.5	0.60	0.03–1	0.25	1	0.36
C. bertholletiae	1	[5]				2				1				0.5
	1	[21]	4				2							
	2	[46]	0.25–0.5			0.35	1–4			2				
Aphophysomyces elegans	6	[9]	0.03–1	0.125			0.03–4	0.125			<0.016–1	0.03		
	1	[5]				2				0.5				0.5
	4	[18]	0.03–1	0.03	0.25	0.33	0.03–8	0.5	2	2.63	0.03–4	0.25	2	1.57
	1	[61]	2				0.5							
Saksenaea vasiformis	4	[18]	0.125–2	0.125	0.25	0.23	0.015–0.03	0.015	0.03	0.05	0.015–0.25	0.06	0.125	0.11
	1	[21]					0.01							
Cokeromyces recurvatus	2	[18]	0.125–2	0.125	2	0.31	0.25–8	0.25	8	4.13	0.25–4	0.25	4	2.13
Syncephalastrum racemosum	2	[46]	0.03			0.03	0.03–0.25			0.1				

n, number of isolates per species; MIC_{50}, MIC causing inhibition of 50% of isolates; MIC_{90}, MIC causing inhibition of 90% of isolates; GM, geometric mean.
A blank space means no data.

Finally, some case reports of successful treatment of patients with zygomycosis have also been published [54–56], highlighting posaconazole as a promising drug for treatment of these infections.

Echinocandins

Echinocandins have been reported as inactive *in vitro* against Zygomycetes [44,46,57]. Caspofungin has been tested against 217 strains [9], all of which were resistant *in vitro* (MICs >16 mg/L). Singh *et al.* [46] found caspofungin to have no activity in a collection of 15 Zygomycetes (MICs >16 mg/L). Kontoyiannis *et al.* [8] also studied the *in vitro* activity of caspofungin against 20 Zygomycetes with similar results (MICs >32 mg/L). However, murine models of zygomycosis [9,58–60] have shown that echinocandins may enhance the activity of AmB in the treatment of these infections. Therefore, echinocandins have potential use when combined with other antifungal drugs.

Terbinafine

Few studies have analysed the activity of terbinafine against Zygomycetes. Dannaoui *et al.* [5] tested terbinafine against 36 Zygomycetes isolates, obtaining a wide range of MICs (Table 1). Terbinafine was active against all isolates of *M. corymbifer* and some *Rhizopus* and *Mucor* isolates. Interestingly, *R. microsporus* was susceptible to the drug, whereas *Rhizopus oryzae* was not.

Combination Therapy

The management of these infections is difficult because of the limited number of drugs active against the causative agents of zygomycosis. Several studies have analyzed the *in vitro* activity of antifungals in combination against Zygomycetes. Dannaoui *et al.* [61] tested 35 isolates of Zygomycetes and found

synergistic effects between terbinafine + AmB and terbinafine + voriconazole (in 20% and 44% of isolates, respectively). Gomez-Lopez *et al.* [62] evaluated the *in vitro* combinations of terbinafine with itraconazole or AmB against 17 clinical isolates of Zygomycetes and found that terbinafine + itraconazole exhibited a synergistic effect in 82% of isolates, especially for *R. microsporus*, *M. corymbifer* and *C. bertholletiae*, as did terbinafine + AmB in 53% of isolates.

Animal models have shown that the interaction between AmB and caspofungin or posaconazole improves survival in mice, indicating a synergistic effect between these drugs [59,63]. Sugar and Liu [39] reported a synergistic effect for the combination of azole drugs and quinolones in mice with pulmonary mucormycosis.

In addition, caspofungin combined with AmB was more successful than AmB alone in treating patients with rhino-orbital-cerebral mucormycosis [60] and this combination was also used successfully to treat a case of rhinocerebral zygomycosis in a haematological cancer patient [64].

Conclusions

Zygomycetes are a heterogeneous group of fungi with a wide antifungal susceptibility profile. Amphotericin B is the agent of choice to treat zygomycosis. However, its toxicity remains a problem and therefore alternative therapies are needed, including, for example, lipid formulations of AmB. Posaconazole is the second most active agent against these fungi and has shown good results *in vitro*, in animal models and also in patients. Combination therapies with azoles or echinocandins may also represent alternatives to improve the survival of patients infected with Zygomycetes.

Transparency Declaration

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3.3.3. Taxonomía y relevancia clínica del género *Lichtheimia*

Species recognition and clinical relevance of the zygomycetous genus *Lichtheimia* (syn. *Mycocladus*, *Absidia* pp.)

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Resumen: El género *Lichtheimia* (syn. *Mycocladus*, *Absidia* pp.) incluye a hongos saprofitos que habitan en la tierra o material vegetal en descomposición. *Lichtheimia corymbifera* (syn. *Absidia corymbifera*, *Mycocladus corymbifer*) y *Lichtheimia ramosa* (syn. *Absidia ramosa*, *Mycocladus ramosus*) pueden causar infecciones graves de rápida evolución principalmente en pacientes inmunodeprimidos. En este estudio se analizan los límites de especie en *Lichtheimia* usando el concepto filogenético de especie basado en el análisis de tres dianas (la región ITS, la región D1-D2 del LSU y parte del gen que codifica para la actina), concepto biológico de especie mediante test de apareamiento, y caracteres morfológicos y fisiológicos. Comparando los dendrogramas producidos mediante la secuenciación de las regiones ITS, LSU y el gen de la actina se reconocieron siete especies filogenéticas distintas. Los resultados de los test de apareamiento no apoyaron el estatus de especie de dos de las siete especies filogenéticas, por lo que se propone la aceptación de cinco especies dentro del género *Lichtheimia*: *L. corymbifera*, *Lichtheimia ornata* comb. nov., *L. ramosa*, *Lichtheimia hyalospora*, y *Lichtheimia sphaerocystis* sp. nov. Sólo las tres primeras parecen tener relevancia clínica. *Lichtheimia blakesleeana* es sinónimo de *Lichtheimia hyalospora* y la especie recientemente descrita *Mycocladus lutetiensis* se reconoce como un sinónimo de *L. ramosa*. Finalmente se aporta una descripción detallada de *L. sphaerocystis* sp. nov. y una clave para la identificación de las cinco especies del género basadas en caracteres morfológicos y el crecimiento a diversas temperaturas

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Species recognition and clinical relevance of the zygomycetous genus *Lichtheimia* (syn. *Absidia* p.p., *Mycocladus*)

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Abstract

The zygomycete genus *Lichtheimia* (syn. *Absidia* p.p., *Mycocladus*) consists of saprotrophic fungi inhabiting soil or dead plant material. *Lichtheimia corymbifera* (syn. *Absidia corymbifera*, *Mycocladus corymbifer*) and *Lichtheimia ramosa* (syn. *Absidia ramosa*, *Mycocladus ramosus*) may cause fulminant infections in patients with impaired immunity. This study investigates species boundaries in *Lichtheimia* using Genealogical Concordance Phylogenetic Species Recognition (using genealogies of ITS, D1/D2 region of the LSU and actin), Biological Species Recognition by mating tests, as well as morphological and physiological characters. The molecular markers used were selected by evaluating polymorphism in nine different loci including β-tubulin, translation elongation factor EF-1α, the largest subunits of the RNA polymerase II (RPB1 and PRB2), the mitochondrial cytochrome c oxidase subunit I (COI), and the mitochondrial ribosomal small subunit (mtSSU) rDNA among four strains belonging to different putative species. Comparing ITS-, LSU- and actin-gene genealogies, we recognized seven phylogenetic species. The results of the mating tests conflicted with two of these. Therefore we regard five species in *Lichtheima* as confirmed: *Lichtheimia corymbifera*, *L. ornata* comb. nov., *L. ramosa*, *L. hyalospora*, and *L. sphaerocystis* sp. nov. Only the first three species seem to have clinical relevance. *Lichtheimia blakesleeana* is reduced to synonymy of *Lichtheimia*

hyalospora and the recently described opportunistic species, *Mycocladus lutetiensis*, is found to be a synonym of *Lichtheimia ramosa*. We provide a detailed description of *Lichtheimia sphaerocystis* sp. nov. and a key for the identification of all herein accepted species based on morphological traits and growth at different temperatures.

Introduction

Mucormycoses, i.e. infections caused by members of the Mucoromycotina are uncommon but often dramatic, requiring immediate action on the basis of accurate diagnosis. The recently observed increase in case reports on mucormycosis (31) can be ascribed to in the growing number of patients with risk factors such as diabetes, neutropenia, bone marrow transplantation, or long-term use of steroids. Several studies particularly relate the increasing incidence of mucormycoses to voriconazole prophylaxis and treatment against aspergillosis infection in immunocompromised patients (18, 36, 42), although this trend was already observed prior to the availability of voriconazole in medical applications (17, 20). According to Roden et al. (31) approximately 5% of mucormycoses are caused by species of *Lichtheimia* (syn. *Absidia* p.p., *Mycocladus*). These authors reviewed 25 well-documented cases of *Lichtheimia* (as *Absidia*) infections since 1940. However, the actual incidence of the species may have been underestimated given the fact that a total of 53 clinical *Lichtheimia* strains were sent to the CBS Fungal Biodiversity Centre (Utrecht, The Netherlands) and the Instituto de Salud Carlos III National Centre of Microbiology (CNM-CM, Madrid, Spain) during the past 10 years alone.

Originally the genus *Absidia* was characterized by the formation of pyriform sporangia with a distinct apophysis and branched sporangiophores. Later phylogenetic and physiological studies showed that *Absidia*-like fungi represent three separate lineages (15): 1 – *Absidia s.str.* that show decreased growth rates above 30°C and no growth above 40°C (mesophilic), and their zygospores are protected by long appendages of the suspensors, 2 – *Lentamyces*, which stops growth at 30°C and consists of potential parasites of other fungi (13), and 3 – *Lichtheimia*, consisting of thermotolerant species that show good growth at human body temperature and produces zygospores with equatorial rings and suspensors without appendages. Only the latter group has clinical relevance. It was first named *Mycocladus* typified by *Mycocladus verticillatus* (15). However, the type of that species turned out to represent a mixed culture of an *Absidia s.str.* and possibly a *Lentamyces* species, thus not congeneric with any of the thermotolerant species.

Therefore this group had to be renamed with the oldest available genus name, *Lichtheimia* (14).

The genus *Lichtheimia* consists of basically saprotrophic species inhabiting soil and decaying plant material. According to Hoffmann et al. (14) the genus *Lichtheimia* contained four species: *L. corymbifera* (syn. *Absidia corymbifera*, *Mycocladus corymbifer*), *L. ramosa* (syn. *A. ramosa*, *M. ramosus*), *L. blakesleeana* (syn. *A. blakesleeana*, *M. blakesleeanus*), and *L. hyalospora* (syn. *A. hyalospora*, *M. hyalosporus*). Of these, only *L. corymbifera* and *L. ramosa* have been reported from human infections. Whether or not *L. ramosa* and *L. corymbifera* are separate species has been a controversial issue in the past, when studies addressing this question applied phenetic criteria. For example, Ellis & Hesseltine (4) treated *L. corymbifera* and *L. ramosa* as being distinct, whereas Nottebrock et al. (27) and Schipper (35) reduced *L. ramosa* to synonymy of *L. corymbifera*. By sequence-analyses of three different loci (ITS, D1/D2 region of LSU, partial EF1-α gene) Garcia-Hermoso et al. (8) found two well-supported clades within a set of strains morphologically identified as *L. corymbifera*. As a result they introduced a novel opportunistic species, *Mycocladus lutetiensis*. Our gene genealogies confirmed the existence of the two groups described by Garcia-Hermoso et al. (8). However, in our study the groups included ex-type strains of the older species *L. corymbifera* and *L. ramosa*, respectively, rendering the name *M. lutetiensis* redundant. In addition, the presence of well-supported subgroups suggested the existence of additional taxa.

The present study aims to explore species boundaries in the genus *Lichtheimia* and to evaluate the clinical importance of each species. Genealogical Concordance Phylogenetic Species Recognition (GCPSR) (39), based on gene genealogies of three loci (ITS, D1/D2 region of LSU, and the actin gene) was used to define phylogenetic species. Mating tests were performed to recognize biological species (Biological Species Recognition), and morphology and growth characteristics were used to develop taxonomic concepts and practical diagnostic features for genus and species. After evaluation of the combined data, we propose to accept five species in *Lichtheimia*, namely *L. corymbifera*, *L. ramosa*, *L. ornata* comb. nov., *L. hyalospora*, and *L. sphaerocystis* sp. nov., of which only the first three are clinically relevant.

Material and methods

Strains

Fifty-three isolates of *Lichtheimia* from environmental and clinical sources, including all ex-type strains of *Lichtheimia* species available at the reference collection of the CBS were included in the study (Table 1). All strains used in this study are either deposited at CBS, at the CNM-CM, or at the Fungal Reference Centre (PRZ, Jena, Germany).

Molecular studies

Extraction of genomic DNA

Cultures were grown for 2 days on malt extract agar (MEA, 5%, malt extract agar produced by Oxoid, Badhoevedorp, The Netherlands) at 24°C. Genomic DNA was extracted according to the procedure described by Möller et al. (24) with several modifications. Briefly, fungal material was transferred to a tube containing 2 glass beads and 500 ml TES buffer (124 mM Tris(hydroxymethyl)-aminomethane, 12.8 mM Na-EDTA, 87 mM sodiumdodecylsulphate (SDS), pH = 8). The samples were homogenized for 3 min at 30 Hz using the TissueLyser (Quiagen, Venlo, The Netherlands) and spined for 2 min at 14,000 rpm (20,400 rcf). Afterwards 5.1 U of Proteinase K (in 10 µl) (Sigma-Aldrich, Zwijndrecht, The Netherlands) were added followed by 30 min of incubation in a waterbath at 55°C. 120 µl of 5 M sodium chloride and 1/10 volume of 10% CTAB (Cetyl trimethylammonium bromide) were added to the material followed by 60 min incubation at 65°C. The samples were again homogenized for 3 min (30 Hz) using the TissueLyser. One volume of SEVAG (chloroform: isoamylalcohol 24:1 v/v) was added and the samples were spined for 5 min at 4°C at 14,000 rpm (20,400 rcf). The upper phase (aqueous phase) was transferred to a new tube and 0.55x volumes of isopropanol were added to precipitate the DNA. After incubation at -20°C for at least 30 min DNA was pelleted at 14,000 rpm (20,400 rcf) for 10 min at 4°C. The supernatant was decanted and the DNA pellet was washed twice with 700 ml 70% ethanol, dried, and resuspended in 50 µl of TE buffer (12.4 mM Tris, 1.34 mM Na-EDTA (pH = 8.0). Genomic DNA was stored at -20°C.

Marker selection

We searched for genomic regions with a polymorphism comparable to that of the ITS region in order to apply GCPSR (39). Using DNA extracts of four strains belonging to different putative species (CBS 100.28, CBS 100.51, CBS 582.65, CBS 958.68) we amplified and sequenced parts of the following genes: actin, β-tubulin, translation elongation factor EF-1α, ITS, D1/D2 region of the nuclear ribosomal large subunit (nucLSU), largest subunit of the RNA polymerase II (RPB1), second largest subunit of the

RNA polymerase II (RPB2), mitochondrial cytochrome c oxidase subunit I (COI), and mitochondrial ribosomal small subunit (mtSSU). The used primers are given in Table 2. All PCR products except of those comprising the LSU and most of them comprising the ITS were cloned before sequencing in the competent cell line JM109 of *E. coli* using the p-GEM-T Easy Vector (Promega, Leiden, The Netherlands) as instructed by the manufacturer. Similarity values based on uncorrected distances for the 6 possible pairings between the 4 strains were calculated for all genomic regions tested with BioNumerics v.4.61 (Applied Maths NV, Sint-Martens-Latem, Belgium). For several loci different sequences for the same strains were obtained. In order to ascertain the origin of this diversity, single-spore cultures of 3 strains (CBS 223.78, CBS 124197, CNM-CM4978) were achieved by suspending sporangiospores in sterile distilled water and plating several dilutions of these suspensions on MEA in Petri dishes. The outgrowing mycelia were isolated. DNA was extracted and used for PCR amplification, cloning and sequencing as described.

Amplification, cloning and sequencing of the selected marker

DNA segments comprising either the complete ITS region and the D1/D2 region of the LSU, or a large part of the actin gene were amplified using the primer sets described above. For both PCR types the reaction mixture (25 µl) contained 0.4 µM of each primer, 0.185 mM of each deoxynucleoside triphosphate (GC Biotech, Alphen a/d Rijn, The Netherlands), 10x NH4 BioTaq Reaction buffer (GC Biotech), a final concentration of 1.5 mM MgCl2, 0.8 U BioTaq DNA polymerase (GC Biotech), and about 20 ng of DNA. The cycling conditions for the ribosomal fragment included one initial cycle at 94°C for 5 min, followed by 35 cycles of 1 min at 94°C, 1 min at 53°C, and 2 min at 72°C, with one final cycle of 7 min at 72°C. For the actin gene fragment the following PCR conditions were applied: predenaturation for 5 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 58°C, and 2 min at 72°C, and one final cycle of 7 min at 72°C. PCR reactions were performed on a Thermal cycler 2720 (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Reactions products were analysed in 1% agarose gels.

Cloning and sequencing

PCR products comprising the actin fragment or the ITS region (in case of CBS 100.36 and CNM-CM 22 4849) were ligated into p-GEM-T Easy Vector (Promega, Leiden, The Netherlands) and cloned in *E. coli* JM109 competent cells (Promega) following the

manufacturer's instructions. Colony-PCRs were performed using the primer pair M13f (5'-GTAAAACGACGGCCAGT-3') and M13r (5'- GGAAACAGCTATGACCATG-3'). In a first step we sequenced 4 clones for each strain. In case that these clones did not include the required outparalog (see results), we sequenced additional clones until all strains were represented by at least one sequence of paralog type I in the alignment. Both strands of the products of the primary PCR (ITS, LSU) or the colony PCR (actin) were directly cycle-sequenced using the Big dye sequencing kit (BigDye Terminator cycle sequencing ready reaction kit, Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and the primer sets mentioned above. Cycle-sequencing products were analysed on an ABI 3730XL automatic sequencer (Applied Biosystems).

Sequence analysis

Consensus sequences were constructed by means of the SeqMan program v.7.2.2 (DNASTAR, Lasergene) and deposited in GenBank under the accession numbers GQ342712-GQ342955 (Table 1). Sequences were aligned using the server version of the MAFFT program (www.ebi.ac.uk/Tools/mafft) and manually corrected in the program Se-Al v2.0a11 (A. Rambaut, 2002; http://tree.bio.ed.ac.uk/software/seal/). In cases where actin gene sequences belonging to the same outparalog of a single strain were different, the most deviating actin gene sequences per strain were included in the alignment. Phylogenetic relationships were estimated for all alignments with a maximum parsimony analysis done in PAUP (Phylogenetic Analysis Using Parsimony) v.4.0b10 (D. L. Swofford, 2002, Sinauer Associates, Sunderland, MA) and with a Bayesian approach using Markov chain Monte Carlo using the computer program MrBayes, v. 3.1.2 (32). In maximum parsimony analyses, heuristic search was performed with 1,000 replicates and tree-bisection-reconnection (TBR) as branch-swapping algorithm. Gaps were treated as missing data. Robustness of the trees was estimated by bootstrap analysis with 1,000 replicates. The Jukes-Cantor-69-model of DNA substitution was selected by the program MrAIC v.1.4.3 (J. A. A. Nylander, 2004, Evolutionary Biology Centre, Uppsala University) for all three alignments and used in the Bayesian analyses. Four simultaneous Monte Carlo Markov chains were run over 2 M generations using random starting trees, and default starting parameters of the DNA substitution model. Trees were sampled every 100 generations. After discarding the first 8 K trees sampled (burn-in), a posteriori probabilities were estimated by computing majority rule consensus trees. *Dichotomocladium elegans* was used as outgroup in analyses of the LSU because it was shown to be closely related

to *Lichtheimia* by O'Donnell (28, as *Absidia* p.p.). The analyses of actin and ITS were performed without an outgroup because inclusion of *Dichotomocladium* decreased the quality of the alignment due to large sequence differences.

Morphological study

Study of culture characteristic and sporangiophore morphology

Strains were cultivated on MEA at 24°C and on Synthetic Mucor Agar (SMA; dextrose, Merck, Amsterdam, The Netherlands; asparagine, Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands, thiamine chloride, Sigma-Aldrich Chemie BV; agar, Oxoid, Badhoevedorp, The Netherlands) described by Chen & Zheng (1) at 28°C in the dark. After 7 days and 3 weeks, respectively, texture and colour of the colonies were described and in case of the newly described species rated using the charts of Rayner (30). Mounts for microscopic examinations of sporangiophore morphology were made from 7-day-old MEA cultures in two different ways for each species, (i) by pressing a piece of adhesive tape in the colony, and (ii) by squashing a small portion of the colony including the submersed mycelium. The fungal material was mounted in lactic acid with cotton blue (2 mg cotton blue ml-1 lactic acid) and 10 in lactic acid only and examined using a microscope of type Nikon eclipse 80i (Nikon, Amstelveen, The Netherlands). Measurements were performed using the software NIS-Elements D 3.0 (Nikon).

Study of the giant cells

In search of additional distinguishing characters we tested development and structure of giant cells on 3 media at different temperatures: on MEA at 24°C, on potato dextrose agar (PDA; dextrose, Merck, Amsterdam, The Netherlands; agar, Oxoid, Badhoevedorp, The Netherlands) at 24°C, 33°C and 37°C, and on yeast extract agar according to Haynes et al. (10) (YEA; yeast extract, Difco, Alphen a/d Rijn, The Netherlands; malt extract, Difco; dextrose, Merck, Amsterdam, The Netherlands; agar, Oxoid, Badhoevedorp, The Netherlands) at 33°C and 37°C. In this first screening five strains of *Lichtheimia corymbifera* (CBS 101040, CBS 120580, CBS 120581, CBS 429.75, CBS 519.71), five strains of *L. ramosa* (CBS 100.55, CBS 103.35, CBS 223.78, CBS 123197, CBS 124198), and all strains of the remaining species (see Table 1) were included. After the first results became available we extended the study on YEA at 37°C to all strains of *L. corymbifera*. Every week the cultures were screened for giant cells after removal of the aerial mycelium

with a sterile needle using a stereomicroscope of type Nikon SMZ 1500 (Nikon, Amstelveen, The Netherlands). Giant cells were taken from the culture and mounted in lactic acid for microscopic examination.

Mating experiments

Sporangiospore suspensions were prepared from 5-day-old cultures grown on MEA in Petri dishes at 24°C by adding roughly 2 ml of sterile distilled water and by sucking the water several times into the pipette. One or two drops of the suspension were placed in a distances of approximately 1-2 cm from the drops of the second strain on YEA as described by Haynes et al. (10) and incubated at 31°C in the dark for 7 weeks. A total number of 168 mating pairs were tested including 73 intraspecific and 95 interspecific mating tests.

Growth kinetics

MEA plates were inoculated with small blocks taken from the edge of 3 day-old colonies. The plates were incubated at the following temperatures: 24°C, 33°C, 37°C, 40°C, 43°C, 46°C, 49°C, and 52°C. The diameter was measured twice a day for 3 days. The diameter of the colony taken 8 hours after inoculation was subtracted from all following measurements. Growth rate, measured in millimeters per hour, was calculated for each strain and temperature. Descriptive and comparative statistical analyses were performed. Comparisons were done by variance analysis (ANOVA) including the Bonferroni post hoc test. A P value < 0.05 was taken as statically significant. Statistical analysis was done with the help of the software package SPSS 16.0 (SPSS S.L., Madrid, Spain). Growth rates of each species at different temperatures were plotted in a graph performed with SigmaPlot 11.0 (SPSS S.L., Madrid, Spain).

Results

Molecular markers for species recognition in *Lichtheimia*

Table 3 lists the similarity values for the analysed genomic regions of six strain pairs. In cases where more than one gene sequence was found per strain for a certain locus, the highest possible similarity value for the respective strain pair is given in Table 3. ITS was by far the most variable among all nine regions tested. The second highest degree of polymorphism was found in the nucleotide sequences of β-tubulin, followed by RPB2, actin, RPB1, LSU, EF-1α, and COI. The mitochondrial SSU was strictly monomorphic. For

some of the studied genes we detected paralogs originating from different duplication events. According to Koonin (19) we distinguish between outparalogs, that evolved via an ancient duplication process preceding speciation, and inparalogs that, at least according to their posistion in gene-geneaologies, might have evolved subsequent to the speciation event. Inparalogs of a certain species are considered as coorthologous to inparalogs of other species if they form a monophyletic clade, which is precondition for using the correct inparalogs for phylogenetic analyses. For β-tubulin, we found several paralogs in the studied strains (CBS 100.28: 4 paralogs, CBS 100.51: 3 paralogs; CBS 958.68: 2 paralogs; CBS 582.65: 3 paralogs) although only 4 clones per strain were sequenced. Paralogs of the same strain showed distinct differences in exonic and extreme differences in intronic sequences. No clear groups were detected in phylogenetic analyses, and the selection of coorthologous paralogs was impossible. For RPB2, we discovered no outparalog in the studied strains. We found 3 inparalogs with comparatively small differences (CBS 958.68: up to 5 basepairs difference between the inparalogs, CBS 582.65: up to 17 basepairs difference between the inparalogs). These conditions would have made the RPB2 gene a promising candidate for species recognition. However, after several attempts we did not obtain editable trace-files for strain CBS 100.28. Most species of *Lichtheimia* possess two outparalogs of actin (I and II), which are easily distinguished and differ in their intronic and exonic sequences. Strains listed as *Lichtheimia blakesleeana* and *L. hyalospora,* and their sibling species *L. sphaerocystis*, a novel species that is described below, deviated in having only outparalog I. For nearly all studied strains we found more than one inparalog originating from the duplication of outparalog I. In all studied species with the exception of *L. ramosa*, inparalogs of the same strain differed by 1 to 6 basepairs. In *L. ramosa* inparalogs of the same strain varied by 13 up to 21 basepairs. Each of the three single-spore isolates of the *L. ramosa* strains CBS 223.78, CBS 124197, and CNM-CM 5111 possessed three different inparalogs originating from outparalog I, suggesting the existence of a gene family. Some of the inparalogs (e.g. clone 2 of CNM-CM4978, clone 1 of CBS 112528) contained single-base deletions in their exonic sequences, which indicates loss of function (pseudogenes). The inparalogs of all *Lichtheimia* species belonging to outparalog I of the actin gene formed a well-supported clade in the phylogenetic tree matching with the coorthologous relation of the inparalogs (Fig. 1b). For RPB1 we found two outparalogs with large sequence and length differences forming two separated clades in phylogenetic analyses. Inparalogs were also present in the RPB1 gene. For strain CBS 582.65 of *L. ramosa* we detected 3 different inparalogs

differing in up to 9 basepairs. For EF-1α, no outparalogs were detected. Inparalogs formed separate clades that consisted of two subclades in some species suggesting at least two duplication events after speciation. However, sequence differences among the inparalogs were high even within the same subclade. The single-spore strain CBS 124197, for example, possessed at least 6 inparalogs of EF-1α with sequence differences of up to 27 basepairs within the same inparalog subclade and up to 40 basepairs between the subclades. No paralogs were detected for the LSU and the COI gene. Our main criteria for the selection of molecular marker for species recognition were variability (Table 3) and, in case of the presence of paralogs, the unambiguous assignment to coorthologous pararlogs. On this basis we chose ITS, actin and LSU.

Molecular phylogenetic analyses

Maximum parsimony analyses based on the ITS alignment (a total of 899 characters, of which 383 were parsimony-informative) resulted in 906 most parsimonious trees (tree length TL = 726 steps), the 14 LSU alignment (a total of 657 characters, of which 78 were parsimony-informative) resulted in 185 most parsimonious trees (TL = 230 steps), and the actin alignment (a total of 885 characters, of which 280 were parsimony-informative) in 228 most parsimonious trees (TL = 653). Tree topologies obtained by maximum parsimony and Bayesian MCMC analyses were largely similar for all three loci and did not contain conflicting well-supported groups. Therefore, the Bayesian trees are not shown but Bayesian posterior probabilities are included in the maximum parsimony trees in Fig. 1. In Fig. 1b the actin tree including outparalog I (blue frame) and II (red frame) is pictured on a small scale. The large scale tree represents only the outparalog I part of the tree (blue frame). We detected only outparalog I in *L. blakesleeana*, *L. hyalospora*, and *L. sphaerocystis*, described below, although we sequenced at least eight clones for each strain. In Fig. 1, strains are attributed the same colour if they belong to a single, well-supported group in the ITS genealogy. Because the same colour coding is used in LSU and actin trees, conflicts in gene genealogies of different loci are visualized by intermixing of colours in supported groups of actin and LSU trees. Tree topologies of ITS, LSU, and actin trees are concordant except for groups of *L. ramosa*. Three groups of *L. ramosa* supported in the ITS genealogy (indicated as vinaceous, light blue, and dark blue in Fig. 1a) are not detected in the LSU and actin genealogies. The group of *L. ramosa* marked in pink in the ITS tree is also supported in the LSU tree but not detected in the actin tree. Actin sequences of three strains (CBS 100.24, CBS 271.65, CBS 582.65) out of four

strains constituting the pink group in the ITS tree form also a supported group in the actin tree. However, none of the thirteen edited actin sequences of CBS 223.78, which represents the fourth strain of the pink group in the ITS tree, belonged to this group. All actin sequences of outparalog I of CBS 223.78 are positioned in another supported group that includes also sequences of CBS 271.65 and CBS 582.65 (pink group). But sequences of the dark blue group of the ITS tree and those sequences that are not part of any supported group in the ITS tree (marked in black) do also cluster in that group of the actin gene tree. We consider the supported group in the actin tree consisting of CBS 100.24, CBS 271.65, and CBS 582.65 as inparalog that is absent in the remaining strains of *L. ramosa*. Therefore, that clade was excluded from the decision about phylogenetic species recognition. Consequently, we regard the pink group of the ITS tree as not detected in the actin tree. Considering that phylogenetic species do not necessarily have to be monophyletic in all loci due to different rates of lineage sorting (16), the following criteria (3) were applied to recognize clades that represent independent evolutionary lineages: 1 – genealogical concordance, i.e. presence in all single-locus genealogies regardless of the level of support, 2 – genealogical nondiscordance, i.e. high support of the clade in at least one single-locus genealogy without supported contradiction in another single-locus genealogy. Only relatively distinct lineages were accepted as phylogenetic species in order to avoid that minor tip clades would have to be recognized as phylogenetic species. Boundaries of phylogenetic species were determined in such a way that no strain was left to be unclassified (3). Applying these criteria, seven phylogenetic species in *Lichtheimia* were recognized (Fig. 1), as follows:

1 – '*L. ramosa* subgroup I' containing the ex-type strains of *Absidia gracilis* (CBS 103.35) and *Absidia idahoensis* var. *thermophila* (As3.4808);

2 – '*L. ramosa* subgroup II' including the ex-type strain of *L. ramosa* (CBS 582.65);

3 – '*L. corymbifera*' including the ex-type strain of *L. corymbifera* (CBS 429.75) and the ex-type strain of *Absidia griseola* (CBS 519.71);

4 – '*L. ornata*' with the ex-type strain of *Absidia ornata* (CBS 291.66); 20

5 – '*L. blakesleeana* subgroup I' containing the ex-type strains of *L. hyalospora* (CBS 173.67), *A.* 21 *blakesleeana* var. *atrospora* (CBS 518.71), and *A. cristata* (CBS 102.36);

6 – '*L. blakesleeana* subgroup II' and containing the ex-type strain of *L. blakesleeana* (CBS 100.36);

7 – '*L. sphaerocystis*' containing 3 strains with a morphology of the sporangiophores similar to that of *L. blakesleeana*.

Judging from ITS sequence data, strain CBS 270.65, which was assigned to the recently described species *Mycocladus lutetiensis* by Garcia-Hermoso et al. (8), belongs to subgroup I of *L. ramosa* (data not shown).

Biological species recognition by successful mating

Zygospores were found in 17 out of 168 mating experiments including 73 intraspecific matings (Fig. 2). Azygospores were observed in a single case (CBS 582.65 x CNM-CM3590). Twelve out of the 17 mating pairs producing zygospores were intraspecific matings and five were interspecific according to taxonomic concepts of *Lichtheimia* maintained in this paper. Mature zygospores developing after intraspecific mating (Fig. 3a-c) were usually dark red brown, (46-) 58-77 (-91) × (38-) 48-67 (-82) µm and possessed 1-3 (-5) equatorial rings. The suspensors were rough and frequently unequal in size. High numbers of zygospores were usually achieved in intraspecific matings. Zygospores resulting from interspecific matings (Fig. 3d-f) were often smaller, less intensively colored (orange brown), and the equatorial rings were frequently less pronounced or absent; they often contained large oil droplets. In three out of five interspecific matings only a small number (< 10) of zygospores was found. Consequently, we consider high numbers of large, darkly pigmented zygospores with distinct equatorial rings as indication of successful mating within the same species. Such zygospores were present in high numbers in several matings between the two *Lichtheimia ramosa* subgroups and in a single mating between the two *L. blakesleeana* subgroups (Fig. 2). Therefore, we regard these subgroups as belonging to the same biological species. In contrast, we consider the phylogenetic species '*L. ornata*' and '*L. sphaerocystis*', as separate species because we did not find conflicting mating results and because both possess morphological traits that distinguish them from their phylogenetic sibling species, *L. corymbifera* and *L. blakesleeana*, respectively (see below).

Morphology

Culture characteristic and sporangiospore morphology

Pigmentation of colony reverse on SMA was used by Hesseltine et al. (11) and Chen and Zheng (1) to distinguish two varieties of *Absidia idahoensis*. Because the ex-type strain of *A. idahoensis* var. *thermophila* was reidentified as *L. ramosa*, we tested the suitability of this character for species identification. However, intraspecific variability in pigmentation of

mycelia and colony reverse did not exceed those between species (data not shown). Therefore this trait is regarded unsuitable for species recognition in *Lichtheimia*.

The morphology of sporangiophores was very similar in *Lichtheimia corymbifera*, *L. ornata*, and *L. ramosa*. Differences in spore shape between *L. corymbifera* and *L. ramosa* were not consistent. Strains with intermediate spore shapes existed in both species (Fig. 4 c,d). Although the majority of *L. corymbifera* strains formed subglobose to broadly ellipsoidal sporangiospores (Fig. 4 a) and many of the *L. ramosa* strains produced ellipsoidal to broadly cylindrical spores (Fig. 4 b) also strains with intermediate spore shapes existed in both species (Fig. 4 c,d). In Fig. 1 the preponderant spore shape in each strain is mapped on the ITS tree demonstrating the inapplicability of this character for species identification. In *L. blakesleeana*, all strains predominantly formed subglobose or more rarely broadly ellipsoidal sporangiospores that differed in size and colour depending on the strains. The two strains constituting subgroup II (CBS 100.28, CBS 100.36) formed small (3.8–5.2 µm diam.), hyaline to brownish, smooth to rough sporangiospores. Subgroup I of *L. blakesleeana* includes the ex-type strain of *A. cristata* (CBS 102.36) producing spores of the same size as subgroup II and two strains with distinctly larger sporangiospores, namely CBS 173.67, ex-type strain of *L. hyalospora*, forming large (4.6–8.3 µm diam.), hyaline to subhyaline, smooth to slightly rough sporangiospores, and CBS 518.71, ex-type strain of *A. blakesleeana* var. *atrospora*, producing large (6.0–11 µm diam.), hyaline to brownish, smooth to rough sporangiospores. Both large-spored strains originate from fermented food and have probably been cultivated and subcultured for a long time. This fact and their grouping with a small-spored strain suggests that *L. hyalospora* and *A. blakesleeana* var. *atrospora* are morphological mutants rather than separate lineages.

Giant cells

Giant cells are strongly swollen, branched or unbranched, often droplet-filled hyphae with thick, refractive walls. Often they were septate especially when branched and in some strains they possessed projections. Giant cells are common in all species of *Lichtheimia*, but their size and complexity depends on medium and growth temperature. On MEA at 24°C numerous strains of all species developed giant cells. They were also common on PDA, where the highest degrees of differentiation were noted at 24°C, and less at 33°C and 37°C. Cultivation on YEA at 37°C was especially appropriate for the discrimination between *L. corymbifera* and *L. ornata*. In *L. blakesleeana*, *L. corymbifera* and *L. ramosa* size and shape of giant cells varied strongly with strain, temperature and medium. They

were slightly or distinctly branched in most strains. Two strains of *L. ramosa* (CBS 223.78 and CBS 124198) had globose, thick-walled giant cells with projections. This type of giant cells is characteristic for *L. sphaerocystis* (see below). *L. ornata* differed from its sister species *L. corymbifera* by forming large [380-760 (-900) × 320-660 (-770) µm], compact, densely branched giant cells (Fig. 9) in two-week-old YEA cultures. They were predominantly formed in the mycelial mat attached to the substrate and in the submerged mycelium, but also occurred, though smaller-sized and less developed, in the aerial mycelium. The agar surface of *L. ornata* isolates appeared characteristically granular on YEA, due to an abundance of large submerged giant cells. In contrast, we could not find any giant cell in *L. corymbifera* in YEA cultures at 37°C.

Growth kinetics

Fig. 5 shows mean growth rate (mm/h) and standard deviations for each species at each temperature analysed. Significant *P* values ($p < 0.05$) were found in all ANOVA analyses performed for each temperature. The largest distinctions between species were found at 40°C and 43°C. At 43°C *L. ramosa* could clearly be distinguished by its growth rate from *L. corymbifera* and *L. ornata* while *L. blakesleeana* and *L. sphaerocystis* did not grow at this temperature. *L. ramosa* was the species with the highest growth velocity at all temperatures. Twenty six out of 30 (86.7%) strains of *L. ramosa* grew at 49°C, but only two strains out of 12 (16.7%) of *L. corymbifera*. According to our experimental design, the maximum growth temperature was 46°C for *L. ornata*, 40°C for *L. blakesleeana*, and 37°C for *L. sphaerocystis* except for a single strain that grew at 40°C.

Synopsis of the results and taxonomic conclusions

On the basis of high numbers of successful matings and lack of diagnostic morphological and physiological characters, we judge that the two subgroups of *Lichtheimia ramosa* represent a single species. Consequently, *Mycocladus lutetiensis* described by by Garcia-Hermoso et al. (8) is a synonym of *L. ramosa*. We also favour to retain the two phylogenetic entities, subgroup I and subgroup II of *L. blakesleeana* within the same species, because of the formation of a high number of large, dark coloured zygospores found in a successful mating between the subgroups, and because of the absence of phenotypically distinctive traits. The basionym of *L. hyalospora* is older than that of *L. blakesleeana* and hence the correct name for the resulting species is *L. hyalospora*. The large-spored strains within this species are considered as mutants because they form a

subgroup with the small-spored environmental strain CBS 102.36 in all three genealogies. The two phylogenetic entities, '*L. corymbifera*' and '*L. ornata*' were distinct and well-differentiated in all three gene-genealogies. In addition, the groups formed different types of giant cells. Therefore, we suggest to maintain *L. ornata* as a separate species. '*L. sphaerocystis*' was also distinct and well-differentiated in all three gene-genealogies and differed morphologically clearly from its sibling species *L. hyalospora* by the formation of consistently globose giant cells. For this reasons we describe it as a new species below. The following taxonomy is proposed (for more synonyms see refs 4 and 12):

Lichtheimia corymbifera (Cohn) Vuill., Bull. Soc. mycol. Fr. 19: 126 (1903).
MycoBank MB416447.
Basionym: *Mucor corymbifer* Cohn, in Lichtheim, Z. Klin. Med. 7: 149 (1884).
Synonyms:
Mycocladus corymbifera (Cohn) J. H. Mirza, in Mirza et al., Mucor. Pakistan (Faisalabad): 95 (1979), comb. inval., Art. 36.1.
Mycocladus corymbifer (Cohn) Vánová, Ceská Mykologie 45: 26 (1991).
Absidia griseola H. Naganishi & Hirahara, in Naganishi & Hirahara, Bulletin of the Hiroshima 20 Jogakuin agricultural College : 13 (1970).

Lichtheimia ramosa (Zopf) Vuill., Bull. Soc. mycol. Fr. 19: 126 (1903).
Mycobank MB416448.
Basionym: *Rhizopus ramosus* Zopf, in Schenk, Handb. Botanik 4: 587 (1890).
Replaced synonym: *Mucor ramosus* Lindt, Arch. Exp. Path. Pharmacol. 21: 269 (1886); nom. illegit., Art. 53.1.
Synonyms:
Mycocladus ramosus (Zopf) J.H. Mirza, in Mirza et al., Mucor. Pakistan (Faisalabad): 95 (1979), comb. inval., Art. 36.1.
Mycocladus ramosus (Zopf) Váňová, Česká Mykologie 45: 26 (1991).
Absidia gracilis Linnem., Flora (Regensburg) 130: 203 (1936); nom. inval., Art. 36.1.
Absidia idahoensis Hesseltine, M. K. Mahoney & S. W. Peterson var. *thermophila* G.-q. Chen & Zheng, Mycotaxon 69: 174 (1998).
Mycocladus lutetiensis Garcia-Hermoso, Gantier & Dannaoui, J. Clin. Microbiol., in press (2009).

Lichtheimia hyalospora (Saito) K. Hoffm., G. Walther & K. Voigt, Mycol. Res. 1: 278 (2009).

MycoBank MB512830.

Basionym: *Tieghemella hyalospora* Saito, Zentbl. Bakt. ParasitKde, Abt. 2, 17: 103 (1906).

Synonyms:

Absidia hyalospora (Saito) Lendn., Mat. Fl. Cryptog. Suisse 3(1): 142 (1908).

Mycocladus hyalospora (Saito) J. H. Mirza, in Mirza et al., Mucor. Pakistan (Faisalabad): 97 (1979), comb. inval.

Absidia blakesieeana Lendn., Bull. Soc. Bot. Genève, Sér. 2, 15: 149 (1924).

Mycocladus blakesleeanus (Lendn.) J. H. Mirza, in Mirza et al., Mucor. Pakistan (Faisalabad): 94 (1979).

Lichtheimia blakesleeana (Lendn.) K. Hoffm., G. Walther & K. Voigt, Mycol. Res. 113: 278 (2009).

Absidia blakesleeana Lendn. var. *atrospora* Schipper, Persoonia 14:141 (1990).

Lichtheimia ornata (A. K. Sarbhoy) A. Alastruey-Izquierdo & G. Walther, comb. nov.

MycoBank MBXXX.

Basionym: *Absidia ornata* A. K. Sarbhoy, Can. J. Bot. 43: 999 (1965).

Synonym: *Absidia hesseltinei* B. S. Mehrotra (as '*hesseltinii*'), Final Technical Report: 48 (1967), nom. inval., Art. 32.1(c).

Material studied: CBS 291.66 (IMI 115180; NRRL 10293), isotype strain, India, Vindhyachal near Allahabad, from dung of bird, isolated by Bot. Dept., Univ. Allahabad, No. M21, 1961, deposited by A.K. Sarbhoy, CBS H-6618; CBS 958.68 (ATCC 24263; NRRL A-11841; VKM F-1524), deposited by C.W. Hesseltine in December 1968, CNM-CM4978, Spain, Aragón, Hospital Miguel Servet, 2007, isolated from a wound of a 50-year-old male.

Lichtheimia sphaerocystis A. Alastruey-Izquierdo & G. Walther, sp. nov.

MycoBank MBXXX, Fig. 6a-i, Fig. 7a-w.

Etymology: Refers to the globose shape of the giant cells.

Latin diagnosis:

Coloniae extensae, cotoneae vel coactae, albae vel cellulis giganteis copiosis cremeae vel sporangiophoris griseae, reversum ochraceum. Temperatura crescentiae optima 33°C, maxima 40°C. Sporangiophora simplicia vel ramosa, singula vel bina orientia, recta vel incurva vel circinata; septum subsporangiale plerumque absens. Sporangia globosa vel pyriformia, multispora, deliquescentia, atrobrunnea vel atra; sporangia maxima terminalia apophysi conica conspicua praedita, 16–43 µm diam; columellae elllipsoideae vel sursum angustatae vel raro subglobosae, rarissime una vel duabus projectionibus praeditae, 8.5–33 × 6.8–29 mm, collari praesente vel absente. Sporangiosporae leves vel asperulatae, hyalinae vel dilute brunneae, acervatae fuscae, subglobosae vel late ellipsoideae vel modice irregulares, 3.5–7 µm diam vel 4.2–6.8 × 3.3–5.5 µm. Cellulae giganteae intercalares, globosae, 60–150 µm diam (strato mucido excluso), saepe guttulatae, crassitunicatae, saepe projectionibus simplicibus vel ramosis praeditae. Stolones et rhizoidea praesentes. Species heterothallica. Zygosporae ignotae.

Colonies expanding on MEA at 24°C, cottony to felty, at first white, later, depending on the proportion of sporangia and giant cells: grey to dark grey (lavender grey to leaden grey according to Rayner 1970) in colonies with predominant sporangiophores, consistently white to cream-coloured in colonies with predominant giant cells, reverse ochreous. On MEA optimal growth temperature 33°C, maximal growth temperature 40°C, no growth at 43°C. Sporangiophores simple or branched, arising solitary or in pairs but not in whorls, either directly from the substrate or from aerial hyphae, hyaline, but often with a light brown apophysis and columella, smooth or slightly rough, straight, bent or circinate. Subsporangial septa mostly absent. Sporangia spherical to pyriform (including apophysis), multi-spored, deliquescing, blackish brown to black, largest sporangia terminal, with conspicuous conical apophysis, 16-43 µm diam. Columella ellipsoidal, ellipsoidal-tapering or more rarely subglobose, occasionally with one or more projections, 8.5-33 × 6.8-29 µm, with or without collar. Sporangiospores smooth to rough-walled, hyaline to light brown, dark brown in mass, subglobose to broadly ellipsoidal or slightly irregular, 3.6-7.0 µm in diameter or 4.2-6.8 × 3.3-5.5 µm. Large intercalary hyphal swellings (giant cells) in aerial hyphae and in the mycelium attached to the medium, but not in the mycelium permeating the medium (substrate mycelium), spherical, 60-150 µm diam. (excluding the mucous layers), septate, often droplet-filled, thick-walled, often with simple or branched projections. Cell wall of the giant cells consisting of 2 refractive layers with a total thickness of 3-14 µm, enclosed by 2 to several mucous layers to 31 µm thick. Projections 9-24 (-40) µm long.

CBS 648.78 ceased to form giant cells after several transfers. Stolons and rhizoids present. Heterothallic. Zygospores not observed.

Holotype: CBS 420.70, India, March 1970, deposited by M.C. Srinivasan.

Other material studied: CBS 647.78, India, Uttar Pradesh, Gorakhpur, dung of mouse, March 1976, isolated and deposited by P.C. Misra, P.C.M. 596; CBS 648.78, India, Uttar Pradesh, Gorakhpur, from soil in *Shorea robusta* forest, March 1977, isolated and deposited by P.C. Misra, P.C.M. 623.

Key to the species

Based on our morphological and physiological results we developed the following key for phenotypic identification of all accepted *Lichtheimia* species. Some characters for the discrimination of *Absidia s.str.* and *Lentamyces* were adopted from Hesseltine & Ellis (12) and Hoffmann et al. (13, 15):

1a. Subsporangial septa present; growth of aerial hyphae indeterminate; most of the sporangiophores in verticils; not thermotolerant; no or reduced growth at 37°C; zygospores with appendaged suspensors.....***Absidia* s.str.**

1b. Subsporangial septa present; aerial hyphae generally ending in a sporangium; verticils of sporangiophores absent; not thermotolerant; no growth above 30°C; homothallic; zygospores warty, without appendaged suspensors.....***Lentamyces***

1c. Subsporangial septa absent or rare; aerial hyphae generally ending in a sporangium; verticils of sporangiophores present in some species but not obvious; thermotolerant; typically good growth at 37°C; heterothallic; zygospores with equatorial rings, without appendaged suspensors.....***Lichtheimia*** (2)

2a. Sporangia dark brown or dark grey to black; colony diameter after 72 h at 43°C < 2 mm; mature sporangiospores rough and/or > 6.5 µm in their longest extension.....3

2b. Sporangia light brownish grey; colony diameter after 72 h at 43°C > 14 mm; mature sporangiospores smooth and < 6.5 µm in their longest extension.....5

3a. Giant cells consistently globose, 60-150 µm diam. (Fig. 6g-i, Fig. 7s-w) .....***Lichtheimia sphaerocystis***

3b. Giant cells (if present) more hypha-like, irregularly swollen, simple to strongly branched, never consistently globose (Fig. 8) .....***Lichtheimia hyaiospora*** (4)

4a. Mature sporangiospores small (< 5.5 µm), rough, and brownish .....small-spored variants of ***Lichtheimia hyalospora***

4b. Mature sporangiospores larger (on the majority > 5.5 µm), smooth or rough, hyaline or brownish.....large-spored variants of ***Lichtheimia hyalospora***

5a. Colony diameter at 43°C after 72 h > 40 mm; spores ellipsoidal to cylindrical or subglobose to broadly ellipsoidal.....***Lichtheimia ramosa***

5b. Colony diameter at 43°C after 72 h < 27 mm; spores never consistently ellipsoidal to cylindrical... 6

6a. Giant cells densely branched (Fig. 9), 380-760 (-900) × 320-660 (-770) µm, present in 2-week-old 6 YEA cultures.....***Lichtheimia ornata***

6b. Giant cells absent from 2-week-old YEA cultures.....***Lichtheimia corymbifera***

Clinical relevance and distribution

Clinical stains are highlighted in red in the ITS tree (Fig. 1a). Judging from these data only species with more pronounced thermotolerance, namely *L. corymbifera*, *L. ornata*, and *L. ramosa*, are clinically relevant. *Lichtheimia corymbifera* and *L. ramosa* seem to be relatively common etiological agents, while *L. ornata* has been isolated only once from a wound of a 50-year-old male. The data suggest similar distribution areas for all clinically relevant species although only strains originating from Asia and Europe were available for studiy and the number of strains was too low to infer geographic distribution.

Discussion

Phylogenetic species recognition and molecular markers

As far as we are aware this is the first study applying GCPSR to a genus of the Mucorales. Multilocus analyses in Mucorales are hampered by the common presence and the high number of paralogs necessitating labour- and cost-intensive cloning steps. Another problem is the limited variability of protein-coding genes compared to the ITS region. Hence, the number of loci used in our study is relatively low for GCPSR. Nevertheless, we consider the resulting species concepts to be reliable because the molecular results are not conflicting among one another, and are furthermore not in conflict with the recognized biological species that are supported by morphological and physiological findings. While most eukaryotes such as animals (25), plants (22), and slime molds (43) are known to have a small actin gene family, fungi were supposed to possess only a single copy for actin, based on results from yeasts like *Saccharomyces cerevisiae* (6) and *Schizosaccharomyces pombe* (23) and filamentous fungi such as *Aspergillus nidulans* (5), *Trichoderma reesei* (21), and *Neurospora crassa* (41). However, recently the zygomycetous species *Abidia glauca* and the basidiomycetous fungi *Schizophyllum commune* and *Suillus bovinus* have been reported to carry two actin-encoding genes (38). Our data suggest the existence of a gene family for actin in the studied species of *Lichtheimia*. The two outparalogs of actin show a high degree of nucleotide polymorphisms including exonic sequences, which suggests a duplication early in or before the diversification of the Mucorales. This fact should be taken into account when using actin for phylogenetic analysis, especially at lower taxonomic levels, because the choice of the outparalog may influence the position of the species in the tree topology. Sequence differences among actin inparalogs originating from the same outparalog were small and could not be explained by conventional divergent modes of evolution. The term 'inparalog' used implicates that the duplication event preceded the speciation. However, cases in which gene copies (repeat copies, inparalogs) in a genome are more similar to each other than to their respective 'orthologous' counterparts (repeats; coorthologous inparalogs) in closely related species could be caused either by the process of by birth-and-death-evolution including recent duplications or by concerted evolution only retaining ancient duplicates (7, 26). In case of concerted evolution the term 'inparalog' would be misapplied. Nevertheless, birth-and-death-evolution is more likely to take place in the actin gene family because it is assumed for many protein-coding gene families (26), and our finding of pseudogenes supports this assumption. Although the presence of several inparalogs is an obvious disadvantage for the use in phylogenetic studies, we think that the use of the actin

gene was legitimate because of the comparatively low sequence variations between actin inparalogs and the lack of an alternative locus.

Biological species recognition by mating test

The relatively low number of successful intraspecific matings (16.4%) corresponds with the results of Ellis and Hesseltine (4), who found only 4.7% of successful matings in *L. corymbifera* (as *Absidia c.*). Successful interspecific matings were previously described for *L. corymbifera* and *L. ramosa* (5) and for *L. ornata* (CBS 958.68, as *Absidia hesseltinei*) and *L. ramosa* (CBS 270.65, as *L. corymbifera*) (35), and also for other genera such as *Mucor* (34). As in the present study, Schipper (34) found zygospores resulting from interspecific mating in the *Mucor racemosus* group to be less coloured and reduced in number and size. Their presence reduces the significance of successful mating for species recognition, even though there is doubt about their occurrence under natural conditions. However, mating test results can still be used to define biological species boundaries when number, appearance, and size of the zygospores are taken into account. Obviously, mating barrages between species in the Mucorales are not as developed as in other fungal groups allowing matings between species that share only 79.8% similarity in their ITS sequences, as in the case of CBS 100.36 x CBS 100.49.

Diagnostic characters

Our results show that sporangiophore morphology and spore shape are insufficient to differentiate *L. corymbifera*, *L. ornata*, and *L. ramosa* and that additional characters are needed. The presence of intermediate spore shapes in *L. corymbifera* and *L. ramosa* may explain why earlier mycologists tended to treat them as synonyms (27, 35). These intermediate spore shapes might also explain why studies that used the spore shape for discriminating both species encountered numerous intraspecific matings (27). Growth rates at certain temperatures as well as appearances of giant cells provided additional valuable characters for species recognition.

Taxonomy

This study shows that *L. corymbifera* and *L. ramosa* are distinct species on the basis of molecular data, as already supposed by Ellis and Hesseltine (4) using morphology and mating tests. *Lichtheimia ornata* was distinguished by the formation of 'peculiar thick-walled globose to elongated bodies with very prominent finger-shaped appendages' (33,

p. 999, as *Absidia ornata*). We consider these structures as early stages of the giant cells that are characteristic for this species although their size described as 44-70 µm is relatively small. Based on morphological features Schipper (35) already recognized *L. sphaerocystis* and *L. hyalospora* (as *L. blakesleeana*) as distinct taxa, referring to strains of *L. sphaerocystis* as '*Absidia* aff. *blakesleeana*'. The degradation of *L. blakesleeana* to a synonym of *L. hyalospora* is supported by the presence of zygospores between strain NRRL 1306, formerly named *A. blakesleeana* f. *hyalospora* and strain NRRL 1306 formerly named *A. blakesleeana* (12, p. 779). However, with respect to *A. blakesleeana* the authors stated on page 782 of the same publication 'the only closely related species is *A. hyalospora* which has much larger spores; it does not seem to fruit on Czapek's solution agar, and it does not mate with *A. blakesleeana* tester strains'. All three isolates of *L. hyalospora* studied by Hesseltine and Ellis (12) were isolated from fermented food ('taosi' or soybeans). Continued subcultivation of single strains for fermentation might have enhanced the occurrence of mutants whose phenotypes can be characterized by an increase in spore size.

In conclusion, by comparing gene genealogies based on three loci (ITS, actin, and LSU) and by considering mating results and differences in morphology and growth rates at different temperatures, we recognize five species in *Lichtheimia*: *L. corymbifera*, *L. ornata*, *L. ramosa*, *L. hyalospora*, and *L. sphaerocystis*. Only the first three species seem to have clinical significance.

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Figure legends

Fig. 1. Maximum parsimony phylograms of three different loci: a. ITS, b. actin [small-scale tree includes outparalog I (blue frame) and outparalog II (red frame), large-scale tree represents only outparalog I (blue frame in the small-scale tree)], c. D1/D2 region of the LSU. Branches with bootstrap values in the MP analysis of 85 or higher are printed in bold. Branch support values are indicated by numbers near the branches (MP bootstrap proportions/Bayesian posterior probabilities) but given only for decisive branches. Strains forming a well-supported group in the ITS phylogram are marked in a certain colour. The same colour is used for each strain also in the LSU and the actin phylogram. In the ITS phylogram, clinical strains are highlighted in red, names are given at the type strains. For each strain of *L. ramosa*, *L. corymbifera*, and *L. ornata* the predominant spore shape is mapped on the ITS phylogram: white/spherical – subspherical to broadly ellipsoidal, subspherical predominant; light grey/broadly ellipsoidal – subspherical to broadly ellipsoidal; medium grey/broadly ellipsoidal - subspherical to broadly ellipsoidal, broadly ellipsoidal predominant; dark grey/ellipsoidal – broadly ellipsoidal to ellipsoidal; black/ellipsoidal – ellipsoidal to broadly cylindrical.

Fig. 2. Results of 73 intraspecific and 95 interspecific matings in *Lichtheimia*. X- no mating test performed, white field – no zygospore formation, grey field – zygospore formation, number of zygospores lower than 10, black field - zygospore formation, number of zygospores higher than 10.

Fig 3. Zygospores of *Lichtheimia*. A-c. intraspecific matings: a. *L. blakesleeana* CBS 100.28 x CBS 102.36, b. *L. ramosa* CBS 124198 x CBS 100.24, c. *L. ramosa* CBS 271.65 x CBS 223.78. D-f. interspecific matings: d. *L. blakesleeana* CBS 100.28 x *L. ornata* CBS 958.68, e. *L. ramosa* CBS 124198 x *L. ornata* CBS 958.68, f. *L. blakesleeana* CBS 100.36 x *L. ramosa* CBS 100.49. Scale bar = 37 50 µm.

Fig. 4. Spore shape in *Lichtheimia corymbifera* (a. and c.) *and L. ramosa* (b. and d.): a. subglobse to broadly ellipsoidal shape of *L. corymbifera* (CBS 429.75 NT), b. ellipsoidal to cylindrical shape of *L. ramosa* (CBS 582.65 NT), c. predominantly broadly

ellipsoidal shape in *L. corymbifera* (CBS 100.31), d. predominantly broadly ellipsoidal shape in *L. ramosa* (CBS 112528).

Fig. 5. Mean growth rate (mm/h) and standard deviations of *Lichtheimia* species at eight different temperatures.

Fig. 6. *Lichtheimia sphaerocystis*, CBS 420.70 (T): a. aerial hypha bearing sporangiophores, scale bar = 25 µm, b-e. sporangiophores with columella, scale bar = 40 µm, f. sporangiospores, scale bar = 20 µm, g. mature giant cell, h. young giant cell; CBS 648.78: i. mature giant cell, scale bar 40 µm.

Fig. 7. *Lichtheimia sphaerocystis*: a. colony surface of CBS 420.70, b. colony reverse of CBS 420.70, 15 c. colony surface of CBS 648.78 (predominant giant cell formation), d. colony surface of CBS 647.78 (predominant sporangiospore formation), e-f. CBS 647.78, sporangiophores, scale bar 50 µm, h-j. CBS 647.78, sporangiophores, k-m. CBS 420.70, part of the sporangiophore with mature sporangium, n-q. CBS 420.70, columella, r. CBS 420.70, sporangiospores, s-v. CBS 420.70, young (s and t) and mature (u and v) giant cells, w. CBS 648.78, mature giant cell, scale bars (h - w) = 20 µm.

Fig. 8. Giant cells of *Lichtheimia hyalospora* formed in PDA cultures: a. CBS 102.36, b. CBS 100.36, scale bars = 100 µm.

Fig. 9. Giant cells of *Lichtheimia ornata* formed in two weeks old YEA cultures: a. younger giant cell formed by CBS 291.66, b. mature giant cell formed by CNM-CM4978, scale bar = 100 µm

1 Table 1. Strains used in this study

2

Strain	Species	Country	Source	GenBank accession no.			
				ITS	LSU	actin	
						outparalog I	outparalog II
CBS 100.17	L. corymbifera	n.a.	n.a.	GQ342885	GQ342942	GQ342820	GQ342715
CBS 100.51	L. corymbifera	n.a.	n.a.	GQ342886	GQ342939	GQ342826; GQ342824	GQ342719
CBS 102.48	L. corymbifera	India	mouldy shoe	GQ342888	GQ342910	GQ342821	GQ342718
CBS 115811	L. corymbifera	Germany	indoor air	GQ342887	GQ342932	GQ342833; GQ342829	GQ342720
CBS 100.31	L. corymbifera	n.a.	aborted cow	GQ342879	GQ342914	GQ342825; GQ342822	GQ342722; GQ342714
CBS 101040	L. corymbifera	France	human; keratomycosis	GQ342882	GQ342918	GQ342830; GQ342819	GQ342723; GQ342721
CBS 109940	L. corymbifera	Norway	human, finger tissue	GQ342881	GQ342917	GQ342817	
CBS 120580	L. corymbifera	France	human; lung	GQ342884	GQ342919	GQ342828	GQ342713
CBS 120581	L. corymbifera	France	human; bronchia	GQ342883	GQ342948	GQ342823	GQ342716
CBS 120805	L. corymbifera	France	human; bone	GQ342880	GQ342915	GQ342818	GQ342717
CBS 429.75	L. corymbifera (NT)	Afghanistan	soil	GQ342878	GQ342903	GQ342831	GQ342712
CBS 519.71	L. corymbifera (T of A. griseola)	Japan	n.a.	GQ342889	GQ342904	GQ342827; GQ342832	

CBS 100.36	L. hyalospora	n.a.	n.a.	GQ342898; GQ342897	GQ342943	GQ342750; GQ342751	
CBS 173.67	L. hyalospora (NT of Tieghemella h.)	Philippines	fermented food taosi	GQ342893	GQ342905	GQ342755	
CBS 102.36	L. hyalospora (T of A. cristata)	Ghana	Manihot esculenta; stem	GQ342895	GQ342907	GQ342752; GQ342753	
CBS 518.71	L. hyalospora (T of M. blakesleeanus var. atrospora)	Japan	n.a.	GQ342894	GQ342944	GQ342754; GQ342756	
CBS 100.28	L. hyalospora (T)	USA	Bertholletia excelsa; nut	GQ342896	GQ342902	GQ342748; GQ342749	
CNM-CM4978	L. ornata	Spain	human; wound	GQ342892		GQ342835	GQ342727
CBS 958.68	L. ornata	n.a.	n.a.	GQ342890	GQ342936	GQ342834	GQ342726
CBS 291.66	L. ornata (T of A. ornata)	India	dung of bird	GQ342891	GQ342946	GQ342836; GQ342837	GQ342724; GQ342725
CBS 100.24	L. ramosa	n.a.	n.a.	GQ342876	GQ342941	GQ342814; GQ342804	
CBS 100.49	L. ramosa	Indonesia	dung of cow	GQ342858	GQ342940	GQ342812; GQ342791	
CBS 100.55	L. ramosa	n.a.	n.a.	GQ342851	GQ342938	GQ342766; GQ342771	
CBS 101.51	L. ramosa	Netherlands	Guinea pig; lung	GQ342859	GQ342945	GQ342796; GQ342789	GQ342747
CBS 101.55	L. ramosa	Switzerlan	human; cornea	GQ342865	GQ342947	GQ342788	GQ342731

		d					
CBS 124198	L. ramosa	Netherlands	culture contaminant	GQ342848	GQ342906	GQ342840; GQ342841	GQ342838
CBS 223.78	L. ramosa	n.a.	cocoa soil	GQ342877	GQ342934	GQ342807; GQ342811	GQ342734; GQ342739
CBS 271.65	L. ramosa	n.a.	n.a.	GQ342875	GQ342937	GQ342805; GQ342816	GQ342746; GQ342740
CBS 649.78	L. ramosa	India	cultivated field soil	GQ342849	GQ342912	GQ342779; GQ342781	GQ342728
CBS 713.74	L. ramosa	n.a.	n.a.	GQ342856	GQ342935	GQ342797	GQ342737
CNM-CM2166	L. ramosa	Spain	human; sputum	GQ342863	GQ342926	GQ342798; GQ342792	
CNM-CM3590	L. ramosa	Spain	human	GQ342869	GQ342924	GQ342785; GQ342786; GQ342810	GQ342741; GQ342744
CNM-CM4119	L. ramosa	Spain	human; skin	GQ342862	GQ342923	GQ342793; GQ342803	GQ342742
CNM-CM4228	L. ramosa	Spain	human; skin	GQ342861	GQ342922	GQ342787; GQ342794	GQ342729
CNM-CM4253	L. ramosa	Spain	human; skin	GQ342860	GQ342921	GQ342780; GQ342795	GQ342733
CNM-CM4261	L. ramosa	Spain	human; lung	GQ342854	GQ342953	GQ342767; GQ342776	
CNM-CM4337	L. ramosa	Spain	human; skin	GQ342852	GQ342920	GQ342765	
CNM-CM4427	L. ramosa	Spain	human, bronchoaspirate	GQ342853	GQ342931	GQ342773	

CNM-CM4537	L. ramosa	Spain	human; skin	GQ342873	GQ342930	GQ342772; GQ342777	
CNM-CM4849	L. ramosa	Spain	human; skin	GQ342855; GQ342868	GQ342929	GQ342769; GQ342778	
CNM-CM5111	L. ramosa	Spain	human; sputum	GQ342871	GQ342928	GQ342783; GQ342784; GQ342806; GQ342808	GQ342735; GQ342736; GQ342743
CNM-CM5171	L. ramosa	Belgium	human	GQ342864	GQ342927	GQ342790; GQ342799	GQ342732
CBS 112528	L. ramosa	Germany	human; wound, double infection with Candida albicans	GQ342850	GQ342913	GQ342764; GQ342813	
CBS 124197	L. ramosa	Greece	human	GQ342870	GQ342951	GQ342842; GQ342843; GQ342844; GQ342845; GQ342846	GQ342839
CBS 269.65	L. ramosa	n.a.	n.a.	GQ342857	GQ342949	GQ342801; GQ342802	GQ342738
CNM-CM1638	L. ramosa	Spain	human; gastric juice	GQ342866	GQ342954	GQ342800	GQ342730
CNM-CM3148	L. ramosa	Spain	human; corneal exudate	GQ342872	GQ342925	GQ342768; GQ342775; GQ342782	

AS 3.4808	L. ramosa (T of A. idahoensis var. thermophila)	China	soil	GQ342867	GQ342955	GQ342770; GQ342774	
CBS 582.65	L. ramosa (NT)	Ghana	Theobroma cacao; seed	GQ342874	GQ342909	GQ342809; GQ342815	GQ342745
CBS 103.35	L. ramosa (T of A. gracilis)	n.a.	Musa sapientum; fruit	GQ342847	GQ342908	GQ342763	
CBS 420.70	L. sphaerocystis	India	n.a.	GQ342900	GQ342933	GQ342760; GQ342761	
CBS 647.78	L. sphaerocystis	India	dung of mouse	GQ342899	GQ342911	GQ342757; GQ342759	
CBS 648.78	L. sphaerocystis	India	soil	GQ342901	GQ342916	GQ342758; GQ342762	

1 Table 2. Primer used for amplification and sequencing of nine different genomic regions in order to

2 select marker for GCPSR.

3

Region	PCR primer	Sequencing primer
actin	Act-1 5'-TGGGACGATATGGAIAAIATCTGGCA-3' (45) Act-4ra 5'-TCITCGTATTCTTGCTTIGAIATCCACAT-3'(45)	Act-1 5'-TGGGACGATATGGAIAAIATCTGGCA-3' (45) Act-4ra 5'-TCITCGTATTCTTGCTTIGAIATCCACAT-3'(45)
β-tubulin	B36f modified 5'-CACCCACTCMCTYGGTGGTG-3' (40) B12r modified 5'-CATGAAGAARTGRAGACGVGGGAA-3'(40)	B36f modified 5'-CACCCACTCMCTYGGTGGTG-3' (40) B12r modified 5'-CATGAAGAARTGRAGACGVGGGAA-3'(40)
COI	M-cox1-fa 5'-GATATGGCATTTCCTCGAT-3'[a] M-cox1-rb 5'-GGWACTGCAATAATCATTGTAGC-3'[a]	M-cox1-fa 5'-GATATGGCATTTCCTCGAT-3'[a] M-cox1-rb 5'-GGWACTGCAATAATCATTGTAGC-3'[a]
EF-1α	MEF-1 5'-ATGGGTAAAGARAAGACTCACG-3' (29) MEF-4 5'-ATGACACCRACAGCGACGGTTTG-3' (29)	MEF-1 5'-ATGGGTAAAGARAAGACTCACG-3' (29) MEF-4 5'-ATGACACCRACAGCGACGGTTTG-3' (29) MEF-11 5'-AAGAAGATTGGTTTCAACCC-3' (29) MEF-21 5'-GGGTTGAAACCAATCTTCTT-3' (29)
ITS	V9G 5'-TTACGTCCCTGCCCTTTGTA-3'(2) LR3 5'-GGTCCGTGTTTCAAGAC-3'(44)	V9G 5'-TTACGTCCCTGCCCTTTGTA-3'(2) ITS1 5'-TCCGTAGGTGAACCTGCGG-3'(46)

		ITS4 5'- TCCTCCGCTTATTGATATGC-3'(46) LS266 5'-GCATTCCCAAACAACTCGACTC-3'(9)
mtSSU	mrSSU1 5'AGCAGTGAGGAATATTGGTC-3' (47) mrSSU3r 5'-ATGTGGCACGTCTATAGCCC-3' (47)	mrSSU1 5'AGCAGTGAGGAATATTGGTC-3' (47) mrSSU3r 5'-ATGTGGCACGTCTATAGCCC-3' (47)
nucLSU	V9G 5'-TTACGTCCCTGCCCTTTGTA-3' (2) LR3 5'-GGTCCGTGTTTCAAGAC-3'(44)	NL1 5'-GCATATCAATAAGCGGAGGAAAAG-3'(28) LR3 5'-GGTCCGTGTTTCAAGAC-3'(44)
RPB1	RPB1-Df modified 5'-TAYAACGCNGATTTCGATGG-3' (37) RPB1-Fr modified 5'-CCTTCACGACCACCCATAGC-3' (37)	RPB1-Df modified 5'-TAYAACGCNGATTTCGATGG-3' (37) RPB1-Fr modified 5'-CCTTCACGACCACCCATAGC-3' (37)
RPB2	RPB2-6f modified 5'-CCYGCWGAAACKCCMGAAGG-3'[b] bRPB2-7r1 5'-CCCATRGCYTGYTTMCCCATDGC-3'[b]	RPB2-6f modified 5'-CCYGCWGAAACKCCMGAAGG-3'[b] bRPB2-7r1 5'-CCCATRGCYTGYTTMCCCATDGC-3'[b]

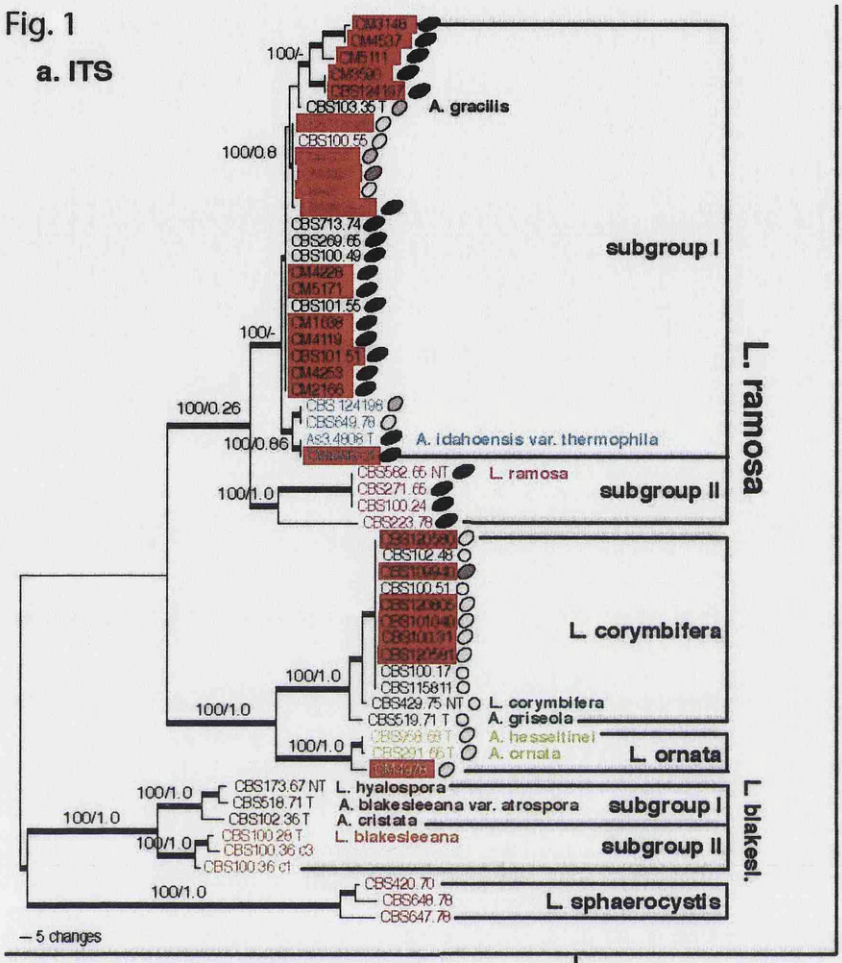
[a] designed for this study.

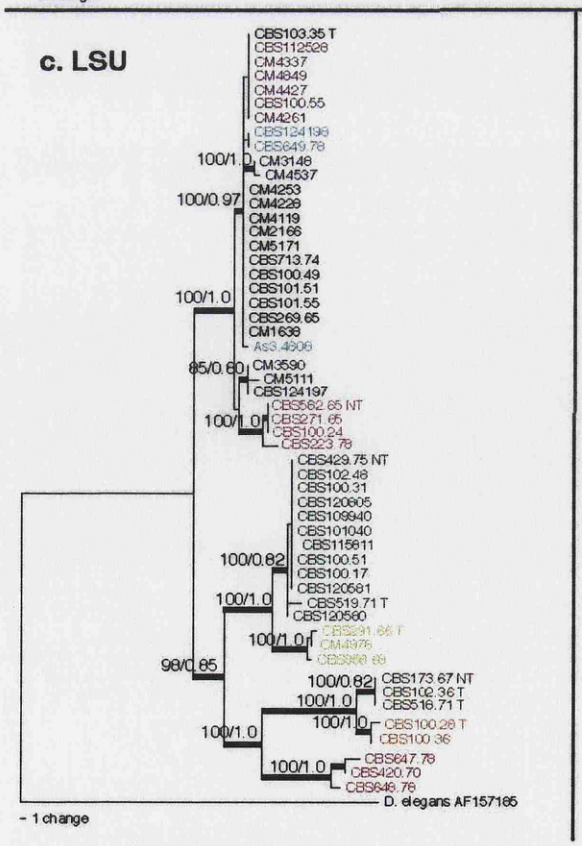
[b] B. Matheny, 2006, PCR primers to amplify and sequence rpb2 (RNA polymerase II second largest subunit) in the Basidiomycota (Fungi) (http://www.clarku.edu/faculty/dhibbett/rpb2%20primers.htm).

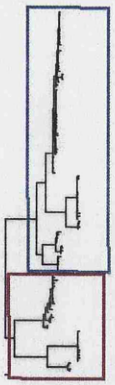
Table 3. Maximal similarity values between six strain pair for nine different loci (ITS, partial β-tubulin, partial RPB2, partial actin, partial RPB1, D1/D2 region of the LSU, partial EF-1α, partial COI, partial mtSSU).

	similarity values in % between the strain pairs								
strain pairs	ITS	ß-tubulin	RPB2	actin	RPBI	LSU	EF-1α	COI	mtSSU
CBS 100.28/CBS 100.51	86.0	91.2	n.a.	91.3	92.4	93.9	94.4	97.8	100
CBS 100.28/CBS 958.68	82.0	90.0	n.a.	91.0	92.6	93.7	94.1	98.2	100
CBS 100.28/CBS 582.65	79.2	91.2	n.a.	94.2	91.1	93.4	94.9	98.4	100
CBS 100.51/CBS 582.65	81.3	88.4	90.0	91.6	91.8	95.5	94.0	97.9	100
CBS 100.51/CBS 958.68	92.4	92.4	97.5	96.2	96.7	98.1	97.7	98.2	100
CBS 582.65/CBS 958.68	81.8	88.1	89.7	92.1	92.1	95.3	93.0	98.1	100

Fig. 1


a. ITS
A. gracilis
A. idahoensis var. thermophila
L. ramosa
subgroup I
subgroup II
L. corymbifera
L. ornata
L. hyalospora
A. blakesleeana var. atrospora
A. cristata
L. blakesleeana
subgroup I
subgroup II
L. sphaerocystis
L. blakesl.
— 5 changes


c. LSU
D. elegans AF157165
- 1 change



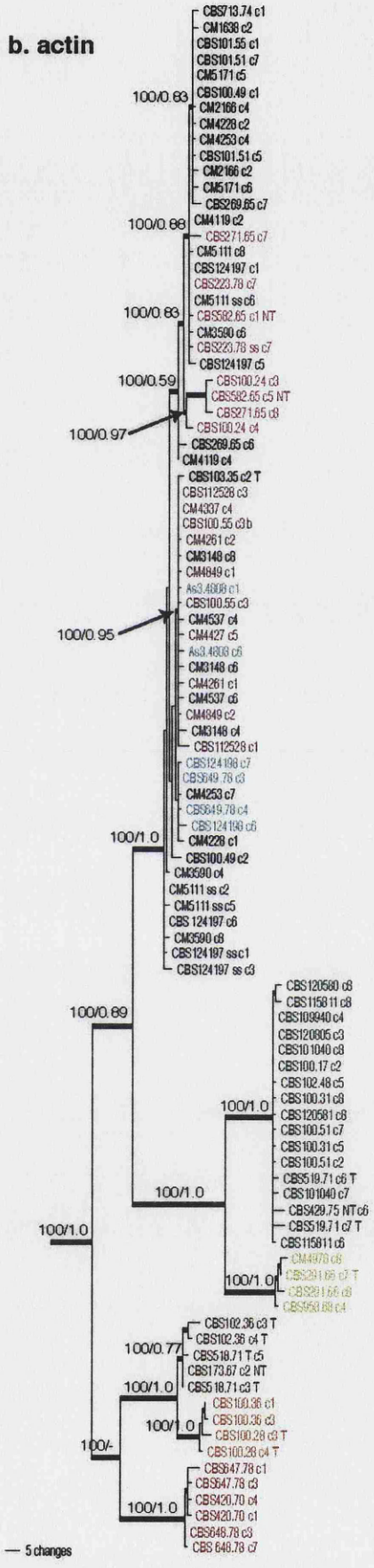

b. actin
— 5 changes

Fig. 2

Species	subgroup	strain number	CBS 173.67	CBS 518.71	CBS 100.28 (T) (+)	CBS 420.70	CBS 647.78	CBS 648.78	CBS 291.66	CNM-CM4978	CBS 100.31 (+)	CBS 100.51	CBS 519.71 (+)	CBS 101040	CBS 120805	AS3.4808	CBS 100.49 (+)	CBS 101.55 (+)	CBS 223.78 (+)	CBS 269.65 (+)	CBS 112528	CBS 124197	CBS 124198 (+)	CNM-CM3590	CNM-CM4119 (+)	CNM-CM4537	CNM-CM4849
L. blakesleeana (T of A. cristata) (-)	I	CBS 102.36			■				X	X	X	X		X	X	X		X	X	X	X	X	X	X	X	X	X
L. blakesleeana f. hyalospora (NT)	I	CBS 173.67	X						X	X	X	X		X	X	X		X	X	X	X	X	X	X	X	X	X
L. blakesleeana (T of A. bl. var. atrospora)	I	CBS 518.71		X					X	X	X	X		X	X	X		X	X	X	X	X	X	X	X	X	X
L. blakesleeana (-)	II	CBS 100.36			■				X	X	X	X		X	X	X	■	X	X	X	X	X	X	X	X	X	X
L. sphaerocystis (T)		CBS 420.70				X																					
L. sphaerocystis		CBS 647.78					X				X	X		X	X	X		X	X	X	X	X	X	X	X	X	X
L. sphaerocystis		CBS 648.78						X	X	X	X	X		X	X	X		X	X	X	X	X	X	X	X	X	X
L. ornata (IT of A. ornata)		CBS 291.66	X	X	X			X	X							X		X	X	X	X	X	X	X	X	X	X
L. ornata (-)		CBS 958.68			■																		■				
L. ornata		CNM-CM4978	X	X	X			X		X						X		X	X	X	X	X	X	X	X	X	X
L. corymbifera		CBS 100.51	X	X	X		X	X			X	X		X	X	X		X	X	X	X	X	X	X	X	X	X
L. corymbifera (NT) (-)		CBS 429.75	X	X	X	X	X	X	X	X						X		X	X	X	X	X	X	X	X	X	X
L. corymbifera		CBS 101040	X	X	X		X	X			X	X		X	X	X		X	X	X	X	X	X	X	X	X	X
L. corymbifera		CBS 120805	X	X	X		X	X			X	X		X	X	X		X	X	X	X	X	X	X	X	X	X
L. ramosa (T of A. idahoensis var. thermophila)	I	AS 3.4808	X	X	X		X	X	X	X	X	X		X	X	X		X	X	X	X	X	X	X	X	X	X
L. ramosa (-)	I	CBS 100.55	X	X	X	X	X	X	X	X	X	X		X	X		■		■				■				
L. ramosa	I	CBS 112528	X	X	X		X	X	X	X	X	X		X	X	X		X	X	X	X	X	X	X	X	X	X
L. ramosa	I	CBS 124197	X	X	X		X	X	X	X	X	X		X	X	X		X	X	X	X	X	X	X	X	X	X
L. ramosa	I	CNM-CM3590	X	X	X		X	X	X	X	X	X		X	X	X		X	X	X	X	X	X	X	X	X	X
L. ramosa	I	CNM-CM4537	X	X	X		X	X	X	X	X	X		X	X	X		X	X	X	X	X	X	X	X	X	X
L. ramosa	I	CNM-CM4849	X	X	X		X	X	X	X	X	X		X	X	X		X	X	X	X	X	X	X	X	X	X
L. ramosa (-)	II	CBS 100.24	X	X	X	X	X	X	X	X	X	X		X	X								■				
L. ramosa (-)	II	CBS 271.65	X	X	X	X	X	X	X	X	X	X		X	X		■		■	■			■				
L. ramosa (NT) (-)	II	CBS 582.65	X	X	X	X	X	X	X	X	X	X		X	X												

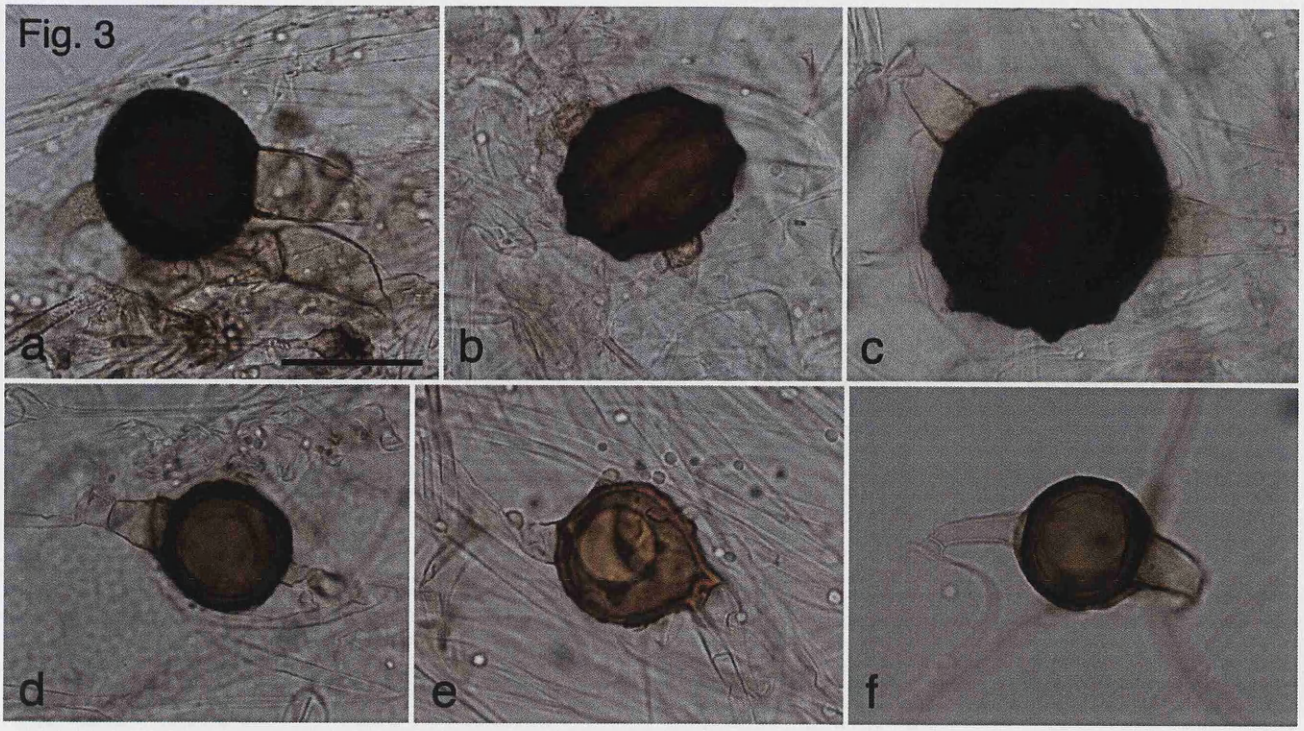
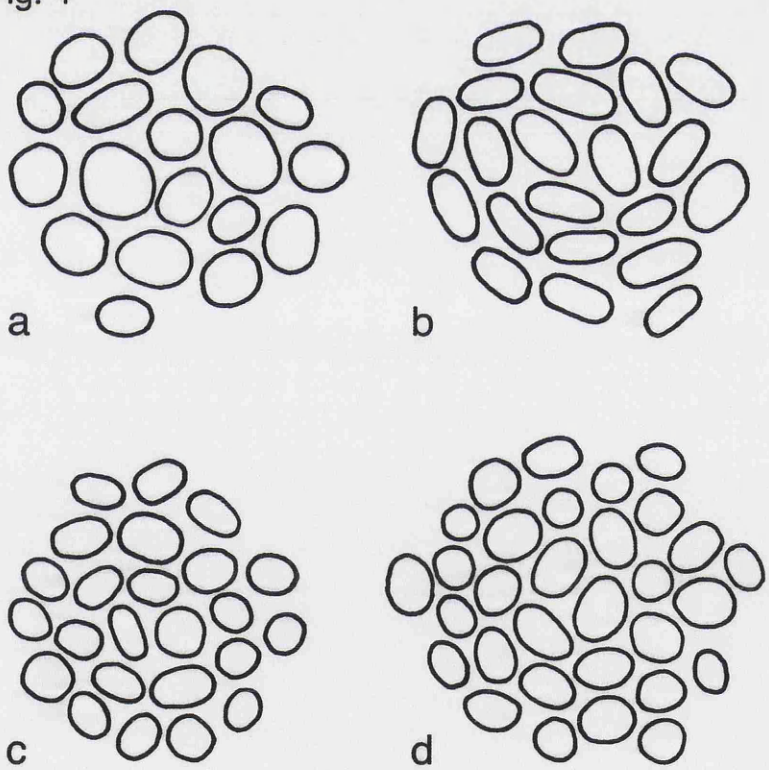


Fig. 4



Growth kinetics

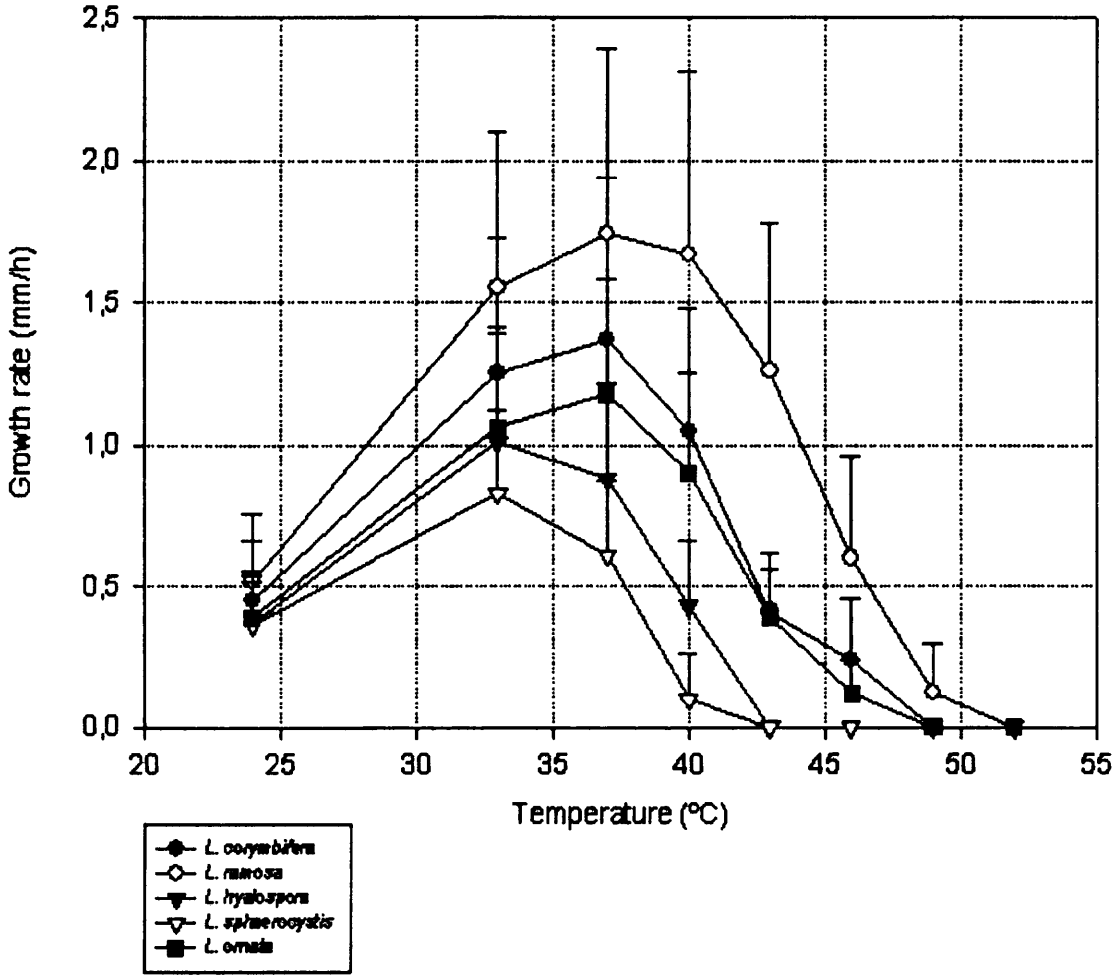
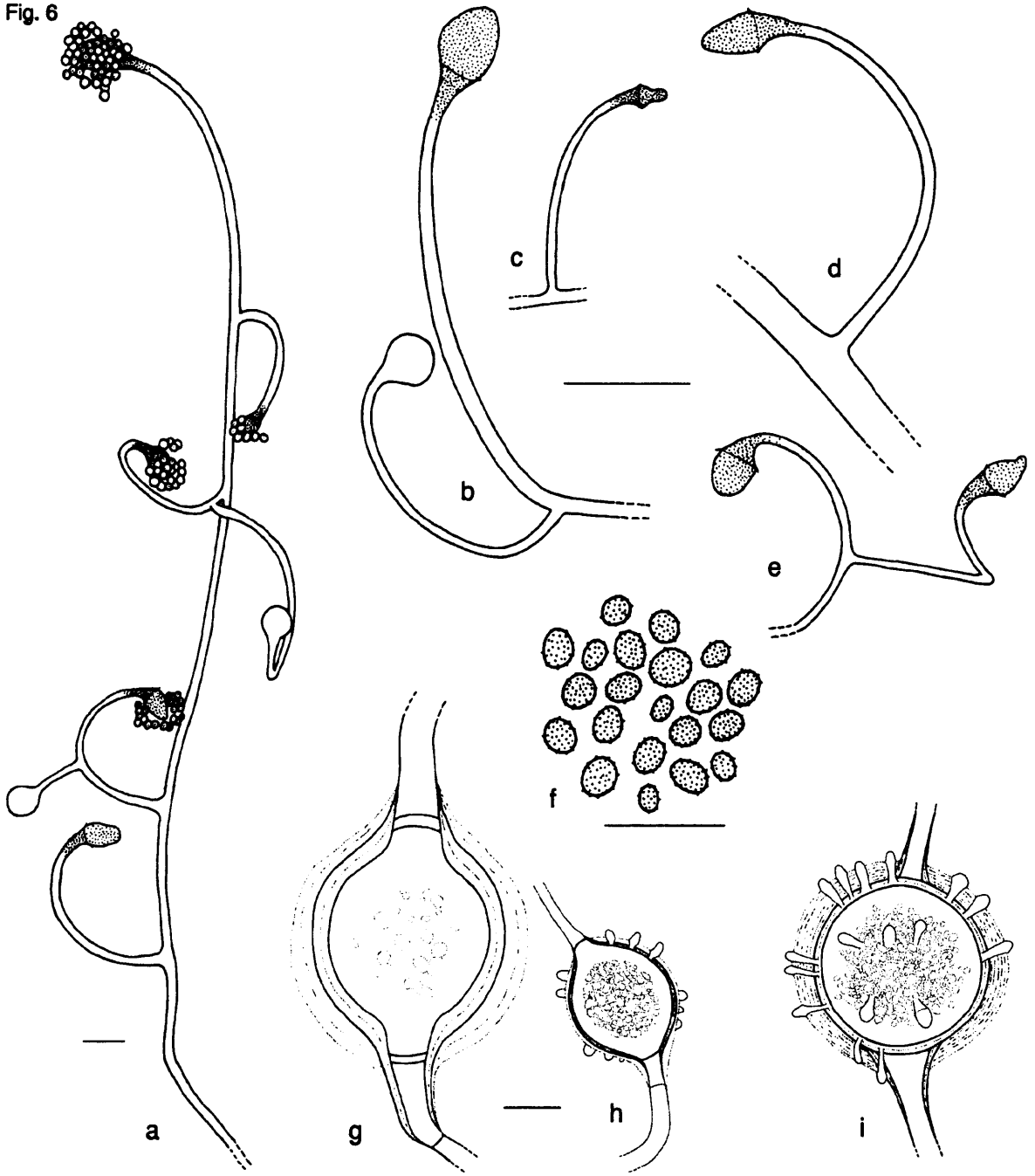


Fig. 6



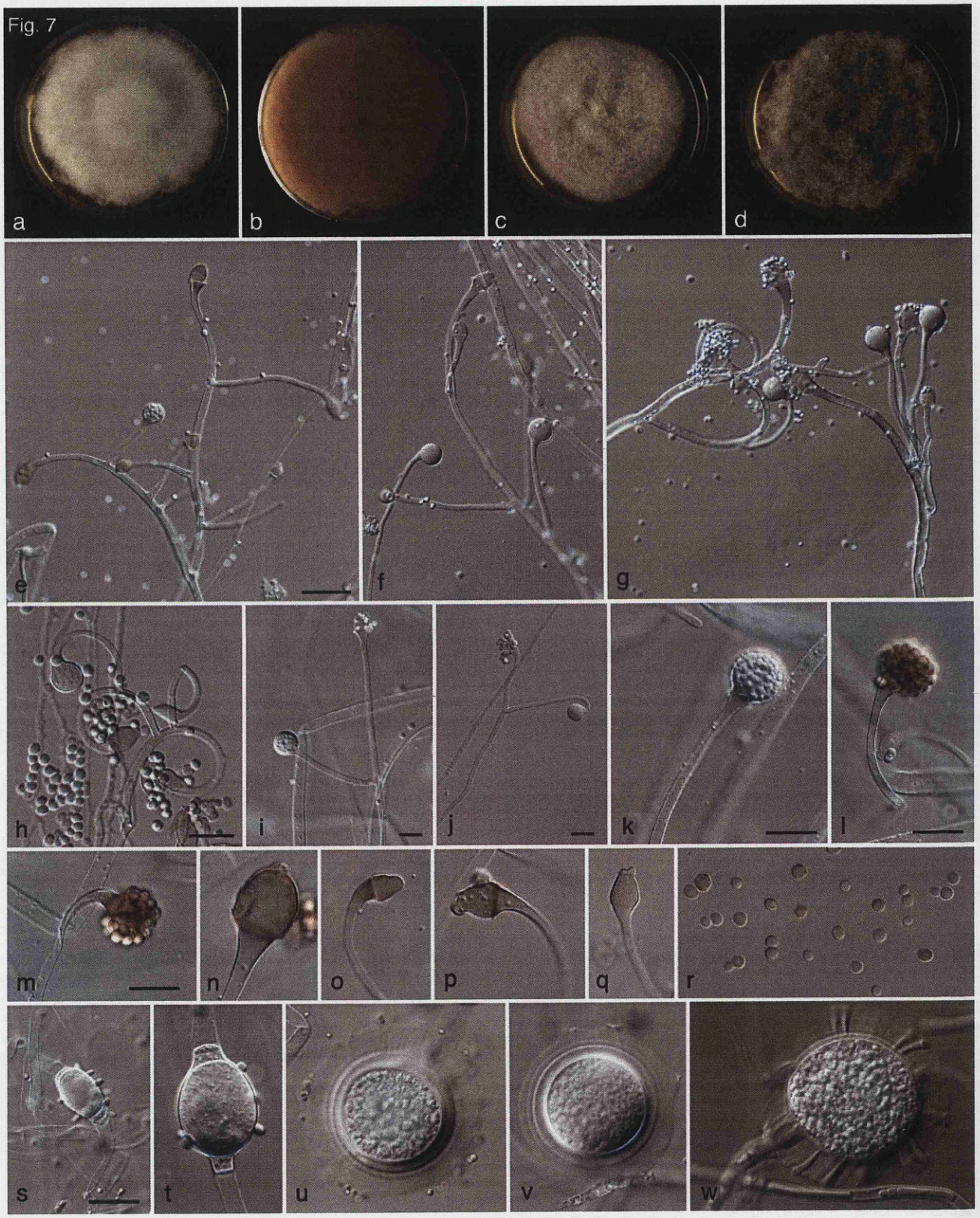


Fig. 7
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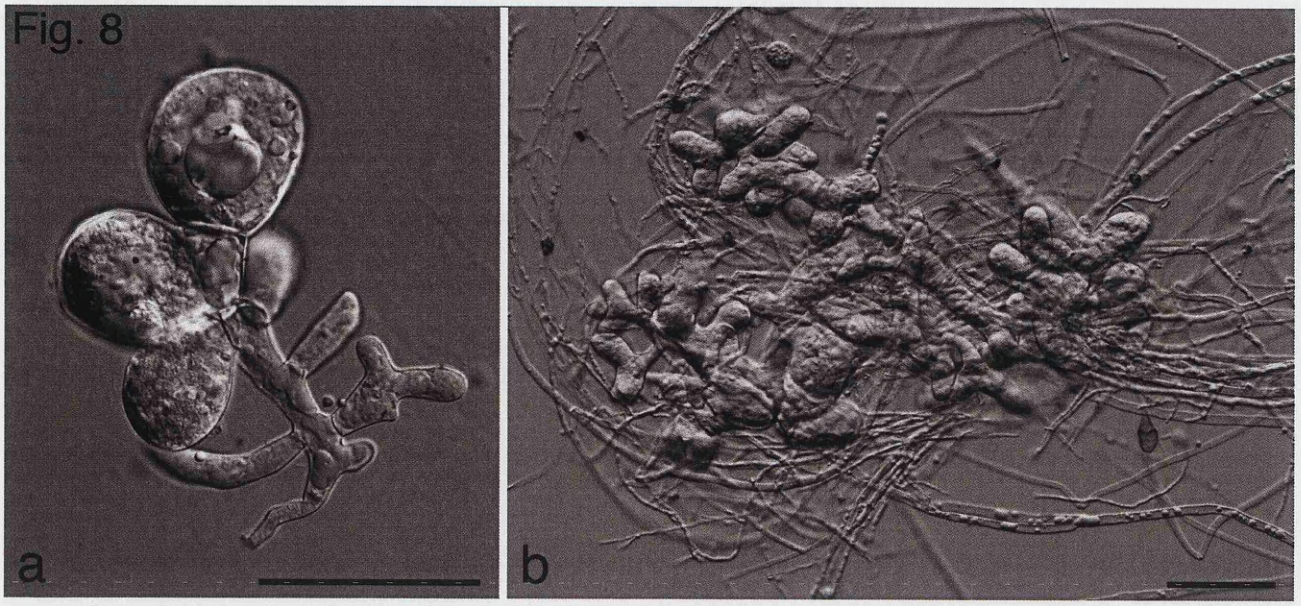
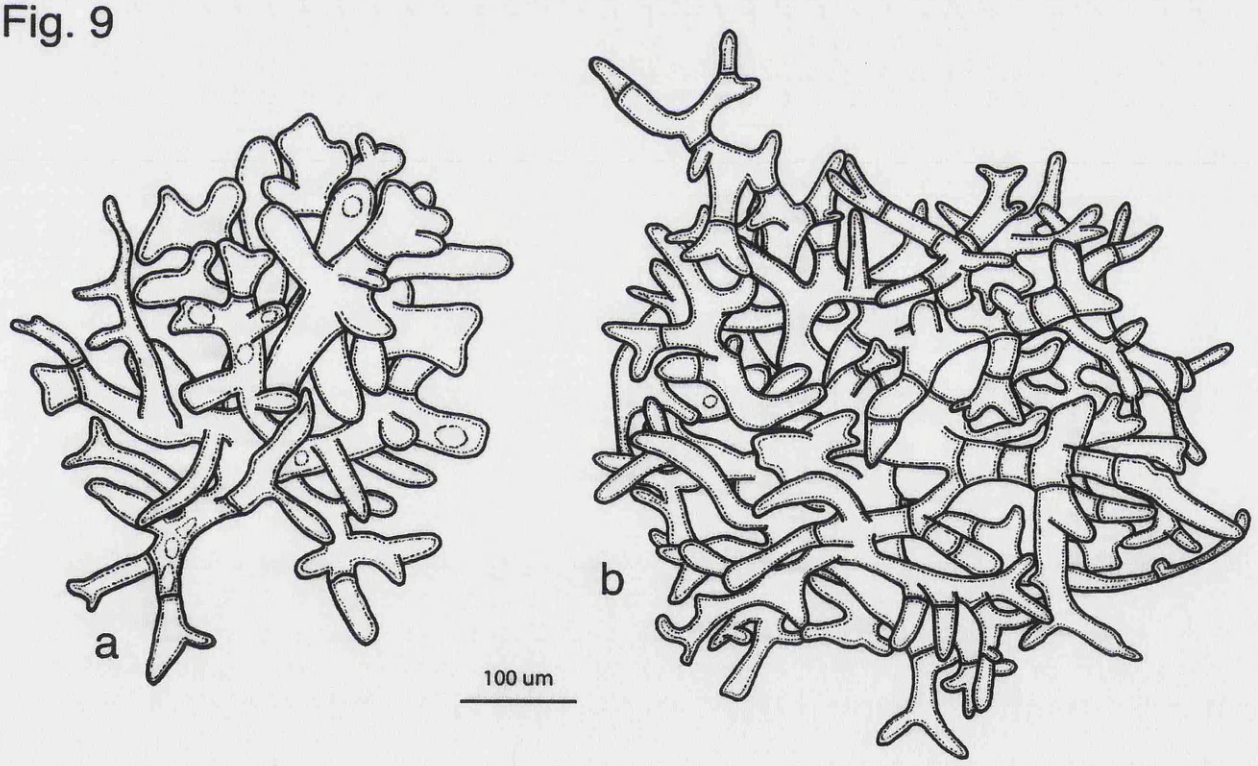


Fig. 9



3.3.4. Perfil de sensibilidad a los antifúngicos de las especies del género *Lichtheimia*

Antifungal susceptibility profile of human pathogenic species of *Lichtheimia*.

Ana Alastruey-Izquierdo[1], Isabel Cuesta[1], Grit Walther[2], Manuel Cuenca-Estrella[1] and Juan Luis Rodriguez-Tudela[1]*

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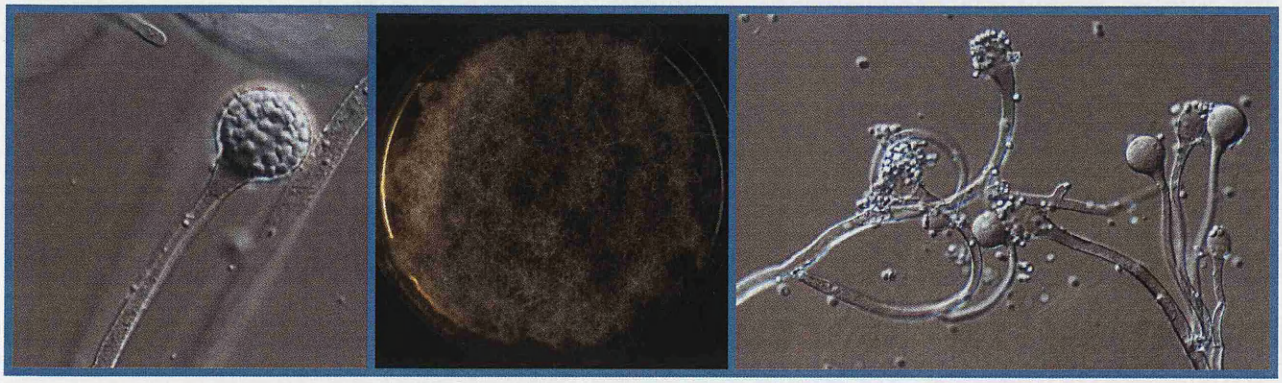
Resumen: Se ensayaron 9 antifúngicos mediante la metodología EUCAST frente a 35 cepas de *Lichtheimia* spp. *Lichtheimia ramosa* mostró CMIs ligeramente mayores para todos los antifúngicos aunque no se encontraron diferencias significativas entre las distintas especies. El posaconazol fue el azol con mayor actividad in vitro mientras que el voriconazol fue inactivo. Las equinocandinas mostraron actividad frente a algunos aislados planteando una alternativa en terapias combinadas.



Antifungal susceptibility profile of human pathogenic species of *Lichtheimia*.

Ana Alastruey-Izquierdo[1], Isabel Cuesta[1], Grit Walther[2], Manuel Cuenca-Estrella[1] and Juan Luis Rodriguez-Tudela[1*]

[1] Servicio de Micologia. Centro Nacional de Microbiologia. Instituto de Salud Carlos III.

[2] CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands

ABSTRACT

Thirty five isolates belonging to human pathogenic species of *Lichtheimia* were tested against nine antifungal agents by using the EUCAST methodology. No remarkable differences were found among the clinical species although *L. ramosa* showed slightly higher MICs for all drugs. Amphotericin B was the most active drug. Among azole drugs, posaconazole had the best activity. Voriconazole was inactive in vitro. Echinocandins showed activity for some isolates suggesting a potential role in combination therapy.

INTRODUCTION

In the last years, the number of cases of zygomycosis (mucormycosis) has increased probably due to the increasing population at risk [16]. The course of zygomycosis infection is rapid progressive and potentially fatal remaining the rates of mortality and morbidity high. Gold standard therapy has not been established yet. It usually requires a combination of measures including antifungal treatment, surgical intervention, and control of the underlying risk factors [15]. The agent of choice for treating this infection is amphotericin B [8]. Posaconazole has been used as salvage therapy for zygomycosis improving the outcome of the patients [9,20]. In addition, echinocandins have been used in combination therapies, pointing out the potential utility of other antifungals in zygomycosis treatment.

The genus *Lichtheimia* is the third most frequent genus isolated in these infections, being responsible of approximately 5% of cases [5,16]. This genus has undergone several taxonomical changes in the last years. Thus, Lichtheimia species were originally classified in the morphologically similar genus *Absidia*. However, molecular phylogenetic analyses revealed their belonging to a separate genus, named *Mycocladus* [10] that had to be renamed in *Lichtheimia* recently [11]. Based on morphological, physiological and molecular data five species were proposed in this genus (Alastruey-Izquierdo et. al.submitted): *Lichtheimia corymbifera*, *Lichtheimia ornata*, *Lichtheimia ramosa*, *Lichtheimia hyalospora*, and *Lichtheimia sphaerocystis*, of

which only the first three are clinically relevant. In the last decades *L. corymbifera* and *L. ramosa* were treated synonymous, and susceptibility values of '*Absidia corymbifera*' or '*Mycocladus corymbiferus*' may refer either to *L. corymbifera* or to *L. ramosa*. In our setting, *L. ramosa* is more common than *L. corymbifera*. in order to know the antifungal susceptibility profile of all clinical *Lichtheimia* species we have analysed the activity of nine antifungal agents against 22 isolates of *L. ramosa* and 12 isolates of *L. corymbifera*. Only one isolate of *L. ornata* was available.

Strains. A total of 35 clinical isolates of *Lichtheimia* species were obtained between 1999 and 2008 in the Mycology Reference Laboratory of the National Centre for Microbiology of Spain. Twenty two strains were identified as *L. ramosa*, 12 as *L. corymbifera* and one isolate as *L. ornata* by means of ITS sequence comparison according to Alastruey et al. (submitted). Fourteen strains were isolated from respiratory sites, six from skin, four from exudates, one from gastric juice, one from peritoneal drainage, and one from hospital air. The source of eight strains was unknown.

Antifungal susceptibility testing. Microdilution testing was performed following the EUCAST standard methodology [19]. Inoculum preparations were performed by means of counting spores in an hematocytometer [1,14,17]. *Aspergillus fumigatus* ATCC 2004305 and *Aspergillus flavus* ATCC 2004304 were used as quality control strains [13].

The antifungal agents used in the study were amphotericin B (Sigma-Aldrich Quimica), itraconazole (Janssen Pharmaceutica, Madrid, Spain), voriconazole (Pfizer S.A., Madrid, Spain), ravuconazole (Bristol-Myers Squibb, Princeton, U.S.A.), posaconazole (Schering-Plough Research Institute, Kenilworth, N.J.), terbinafine (Novartis, Basel, Switzerland), caspofungin (Merck & Co., Inc., Rahway, N.J.), micafungin (Astellas pharma Inc., Tokio, Japan) and anidulafungin (Pfizer S.A.). The final concentrations tested ranged from 16 to 0.03 mg/L for amphotericin B, terbinafine, caspofungin, micafungin and anidulafungin, and from 8 to 0.015 mg/L for itraconazole, voriconazole, ravuconazole and posaconazole. The plates were incubated at 35ºC for 48 h in a humid atmosphere. Visual readings were performed at 24 and 48 hours with the help of a mirror. The endpoint for amphotericin B, itraconazole, voriconazole, ravuconazole, posaconazole and terbinafine was the antifungal concentration that produced a complete inhibition of visual growth at 24 and 48 hours. For the echinocandins the endpoint was the antifungal concentration that produces a visible change in the morphology of the hyphae compared with the growth control well (minimum effective concentration, MEC) [6,12].

Table 1 shows the geometric mean (GM) of MICs and range for the three *Lichtheimia* species identified by molecular methods. Since the time plays a critical role in the management of these infections and most members of the Mucorales are fast growing fungi, it has been recommended to give MICs results for this fungal group at 24 hours [4]. However, we provide results at both times because it is still not clear if all resistant strains can be detected after 24 hours. Although no remarkable differences were found between *L. corymbifera* and *L. ramosa*, the latter species showed slightly higher MICs to most drugs. The highest differences were found for itraconazole (GM of MICs of *L. ramosa* 2.66 mg/L and 0.89 mg/L for *L. corymbifera* at 48 hours of incubation). MICs of *L. ornata* were only based in one isolate, therefore no conclusions could be obtained and more isolates are needed to evaluate the action of antifungals against this specie. Amphotericin B was the most active drug, showing a GM of 0.07 mg/L at 24 hours. Among the azoles voriconazole was invariably inactive against all isolates whereas posaconazole had the highest activity (GM of MICs of 0.33 mg/L at 24 hours). Although itraconazole has shown poor activity against most zygomycetes [3] several isolates of *Lichtheimia* showed low MICs to this drug. These results are in accordance with previous reports where *L. corymbifera* showed the lowest MICs to itraconazole among zygomycetes species [2,7,18]. Terbinafine showed low MICs for most of the strains, although some isolates had MICs higher than 2 mg/L. Regarding echinocandins, two out of 12 strains of *L. corymbifera* and three out of 22 of *L. ramosa* showed low MICs at 24 hours. Anidulafungin was the echinocandin showing the best activity especially against *L. ramosa* where 10 out of 22 strains showed MICs < 2 mg/L, pointing out the potential utility of this drug in combination therapies against this species. More data are needed in order to obtain a clear picture of susceptibility profile and clinical importance of these species. Little is known about their prevalence, and there are no studies regarding epidemiology, pattern of disease, risk factors, etc. We strongly recommend sending all strains of *Lichtheimia* species involved in human infections to reference laboratories where those isolates can be properly identified to species level and where antifungal susceptibility testing can be performed. In such way, the importance of these species could be ascertained.

Acknowledgements

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Table 1. Antifungal susceptibility results of clinical isolates of *Lichtheimia* spp.: MIC GMs and ranges

Specie (number of isolates)	AMB		ITC		VCZ		RVC		PSC		TRB		CAS		MIC		AND	
	24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48
Lichtheimia (35)																		
MG	0.07	0.13	0.80	1.77	12.27	13.59	1.27	2.62	0.33	0.63	0.45	0.87	9.62	15.56	6.87	16.74	2.78	6.67
Range	0.015-0.5	0.015-0.5	0.06-16	0.12-16	2-16	2-16	0.06-8	0.12-16	0.12-16	0.12-16	0.03-16	0.06-16	0.015-32	0.015-32	0.015-32	0.015-32	0.015-32	0.015-32
L. ramosa (22)																		
MG	0.06	0.14	1.00	2.66	12.29	14.96	1.49	3.61	0.32	0.71	0.52	1.00	9.74	20.39	5.82	20.39	3.53	5.10
Range	0.015-0.5	0.015-0.5	0.25-8	0.5-16	4-16	8-16	0.5-4	1-8	0.125-0.5	0.25-2	0.03-2	0.06-4	0.03-32	0.015-32	0.015-32	0.015-32	0.03-32	0.015-32
L. corymbifera (12)																		
MG	0.06	0.12	0.56	0.89	11.99	11.31	1.00	1.59	0.35	0.56	0.37	0.71	8.46	9.59	7.94	11.63	1.71	9.33
Range	0.015-0.25	0.06-0.25	0.06-16	0.125-16	2-16	2-16	0.06-8	0.125-16	0.125-16	0.125-16	0.06-16	0.25-16	0.015-32	0.03-32	0.015-32	0.03-32	0.015-32	0.25-32
L. ornata																		
CNM-CM 4978	0.12	0.12	0.5	1	16	16	1	2	0.25	0.25	0.25	0.5	32	32	32	32	4	32

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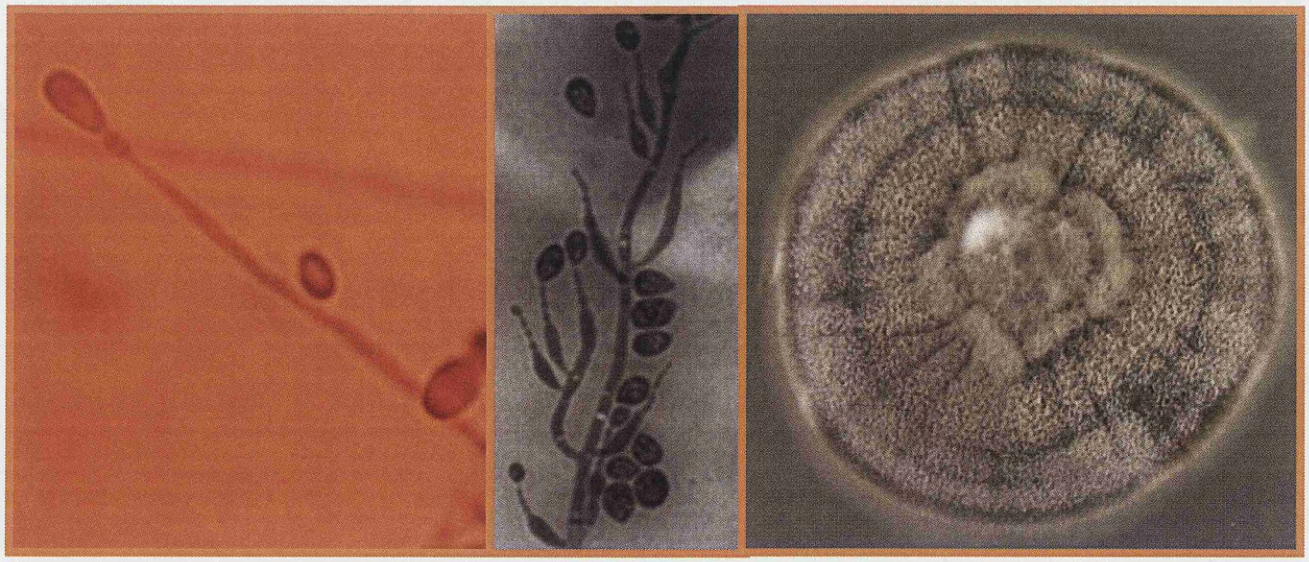
3.4. *Scedosporium* spp.

3.4.1. Prevalence and susceptibility testing of new species of *Pseudallescheria* and *Scedosporium* in a collection of clinical mold isolates.

Antimicrobial Agents and Chemotherapy 2007; 51(2):748-751.

3.4.2. In vitro activities of 35 double combinations of antifungal agents against *Scedosporium apiospermum* and *Scedosporium prolificans*.

Antimicrobial Agents and Chemotherapy 2008; 52(3):1136-1139.



3.4.1. Análisis de las nuevas especies de *Psudallescheria* y *Scedosporium*

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 2007, p. 748–751 Vol. 51, No. 2
0066-4804/07/$08.00+0 doi:10.1128/AAC.01177-06

Prevalence and Susceptibility Testing of New Species of *Pseudallescheria* and *Scedosporium* in a Collection of Clinical Mold Isolates[▽]

Ana Alastruey-Izquierdo, Manuel Cuenca-Estrella, Araceli Monzón, and Juan L. Rodriguez-Tudela*
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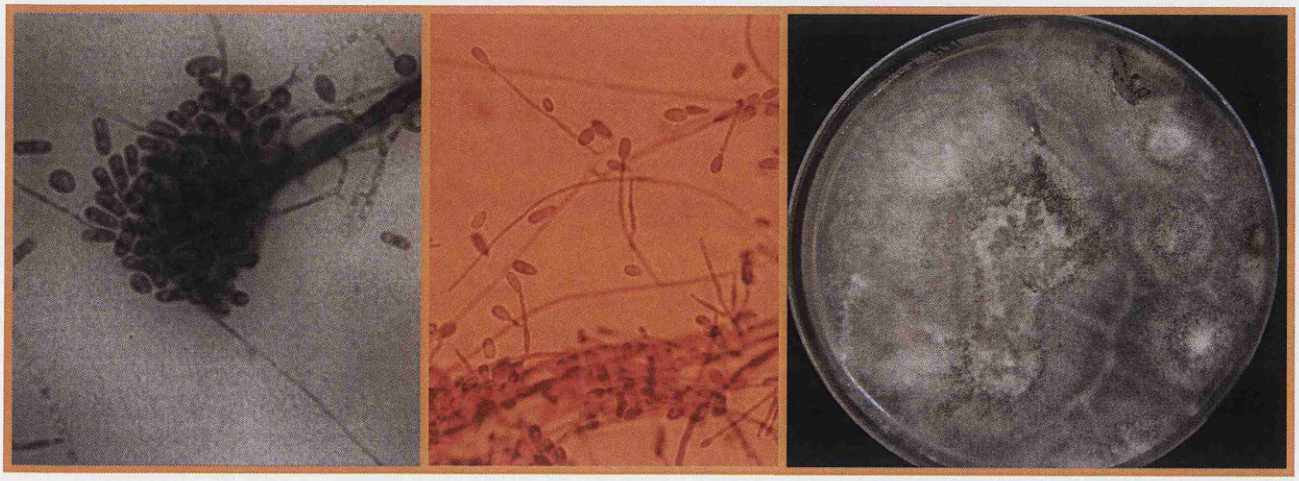
Received 21 September 2006/Returned for modification 21 October 2006/Accepted 4 November 2006

Resumen: Se analizó la prevalencia de las nuevas especies de *Pseudallescheria* y *Scedosporium* en una colección de 46 aislados clínicos. La identificación se hizo mediante métodos morfológicos y moleculares. Se detectaron cuatro *Scedosporium aurantiacum.* El perfil de sensibilidad de *S. aurantiacum* fue similar al de *S. apiospermum.*



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Prevalence and Susceptibility Testing of New Species of *Pseudallescheria* and *Scedosporium* in a Collection of Clinical Mold Isolates[▽]

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Received 21 September 2006/Returned for modification 21 October 2006/Accepted 4 November 2006

The prevalence of new species of *Pseudallescheria* and *Scedosporium* in a collection of 46 clinical isolates was analyzed. Strain identification was done by morphological and molecular methods. Four *Scedosporium aurantiacum* isolates were detected among the panel of clinical strains. The susceptibility profile of *S. aurantiacum* was similar to that of *Scedosporium apiospermum*.

Pseudallescheria boydii is a pathogen able to cause asymptomatic colonization and localized and disseminated infections (11).

Recently, it has been demonstrated that high genetic variation exists in the *P. boydii* species complex (2, 4, 8). Two new species, *Pseudallescheria minutispora* and *Scedosporium aurantiacum*, are phylogenetically and morphologically separated from *P. boydii*. In addition, *P. angusta*, *P. ellipsoidea*, and *P. fusoidea* seem to present genetic differences and could be proposed as new species of *Pseudallescheria* (4).

We have analyzed the prevalence of these new species and their antifungal susceptibility profiles in a collection of clinical isolates of *P. boydii*.

Strains. A total of 46 clinical isolates of *P. boydii* and *S. apiospermum* were included in this study. Twenty-four strains were isolated from respiratory sites, five from biopsies, six from ear samples, five from skin, four from ocular samples, one from blood culture, and one from an abscess.

Table 1 displays the identification of the 42 sequences obtained from the GenBank database that were used as controls.

Morphological identification. All isolates were identified by conventional methods (3).

PCR and DNA sequencing of the internal transcribed spacer (ITS) region. Molds were cultured in GYEP medium (0.3% yeast extract, 1% peptone [Difco, Madrid, Spain], 2% glucose [Sigma Aldrich Química, Madrid, Spain]) for 24 to 48 h at 30°C. Genomic DNA was isolated using a previously described extraction procedure (5).

DNA segments comprising the ITS1 and ITS2 regions were amplified with primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (1) in a GeneAmp 9700 PCR system (Applied Biosystems) (10). The reaction products were analyzed in a 0.8% agarose gel.

Sequencing reactions were done with 2 μl of reaction mixture from a sequencing kit (BigDye terminator cycle sequencing kit, Ready Reaction mixture; Applied Biosystems), 1 μM of the primers (ITS1 and ITS4), and 3 μl of the PCR product in a final volume of 10 μl.

Sequence analysis. Sequences were assembled and edited using the SeqMan II and EditSeq software packages (Lasergene; DNAstar, Inc., Madison, WI). Sequence analysis was performed by comparison of the DNA sequences with

TABLE 1. *Scedosporium* reference strains used for comparison of ITS sequences and their GenBank accession numbers

Species	Strain identification	GenBank accession no.
Pseudallescheria angusta		AJ888414
Pseudallescheria angusta		AJ888413
Pseudallescheria ellipsoidea	CBS418,73	AJ888426
Pseudallescheria ellipsoidea		AJ888427
Pseudallescheria fusoidea	CBS106,53	AJ888428
Pseudallescheria fusoidea		AJ888429
Pseudallescheria minutispora		AJ888424
Pseudallescheria minutispora		AY228119
Pseudallescheria minutispora		AJ888384
Scedosporium apiospermum		AJ888443
Scedosporium apiospermum		AJ888392
Scedosporium apiospermum		AF181558
Scedosporium apiospermum		AJ888385
Scedosporium apiospermum		AJ888438
Scedosporium apiospermum		AJ888391
Scedosporium apiospermum		AY213683
Scedosporium apiospermum	CBS 10854	AY228112
Scedosporium apiospermum		AJ888400
Scedosporium apiospermum		AY217658
Scedosporium apiospermum		AY228123
Scedosporium apiospermum		AY934802
Scedosporium apiospermum		AF455484
Scedosporium apiospermum	CBS 10122	AY213680
Scedosporium apiospermum		AY213681
Scedosporium apiospermum	CBS 59190	AY228118
Scedosporium apiospermum		AY228122
Scedosporium apiospermum		AY213682
Scedosporium apiospermum		AJ888398
Scedosporium apiospermum		AJ888436
Scedosporium apiospermum		AJ888386
Scedosporium apiospermum		AJ888387
Scedosporium apiospermum		AJ888394
Scedosporium apiospermum		AJ888397
Scedosporium apiospermum		AJ888405
Scedosporium apiospermum		AJ888410
Scedosporium apiospermum		AJ888411
Scedosporium apiospermum	CBS101,22	AJ888435
Scedosporium apiospermum		AJ888437
Scedosporium aurantiacum		AJ888432
Scedosporium aurantiacum		AJ888439
Scedosporium aurantiacum		AJ888440
Scedosporium aurantiacum		AJ888441

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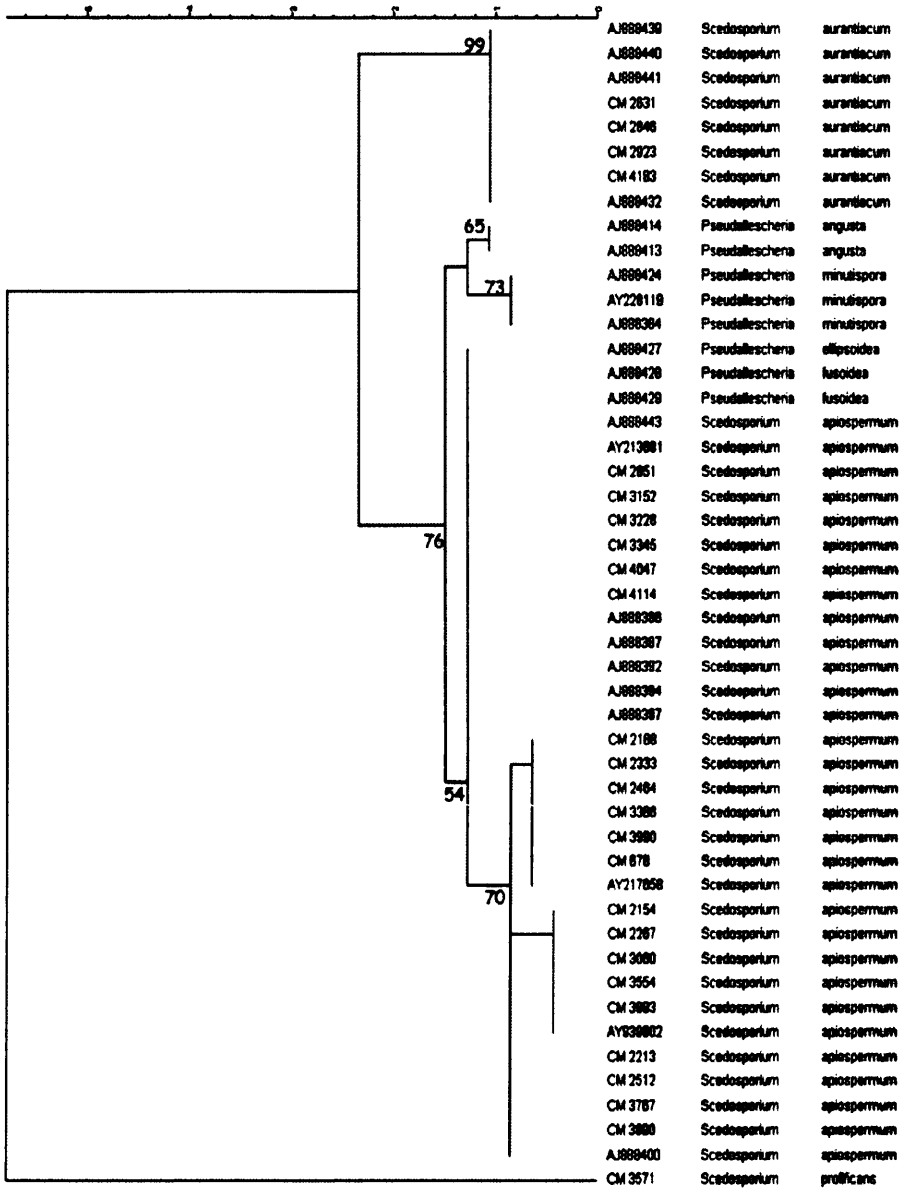

FIG. 1. Phylogenetic tree of the subset of isolates included in the study obtained by using maximum parsimony phylogenetic analyses and 2,000 bootstrap simulations based on ITS sequences. *Scedosporium prolificans* CNM-CM-3751 was used as the outgroup to root the tree.

42 ITS sequences of *Scedosporium* and *Pseudallescheria* strains obtained from the GenBank database (http://www.ncbi.nih.gov/GenBank/). Full information about these strains is displayed in Table 1.

Phylogenetic analysis. All phylogenetic analyses were conducted with Fingerprinting II Informatix software, version 3.0 (Bio-Rad Laboratories, Madrid, Spain). The methodology used was maximum parsimony clustering. Phylogram stability was assessed by parsimony bootstrapping with 2,000 simulations. The ITS sequence of *Scedosporium prolificans* CNM-CM-3571 (Mold Collection of the Spanish National Center for Microbiology) was used as the outgroup.

Antifungal susceptibility testing. Microdilution testing was performed following the CLSI (formerly NCCLS) reference method (6) with the following minor modifications. (i) RPMI 1640 was supplemented with glucose to reach a 2% concentration. (ii) Inoculum size was between 1×10^5 to 5×10^5 CFU/ml. Inoculum preparations were performed by means of

TABLE 2. Antifungal susceptibility results for clinical isolates of *Scedosporium aurantiacum* and geometric mean and range of the 42 *Scedosporium apiospermum* isolates

Isolate	Strain no.	Sample	MIC (mg/liter)		
			Amphotericin B	Itraconazole	Voriconazole
S. aurantiacum	CNM-CM-2923	Corneal	8	16	16
	CNM-CM-2631	Sputum	4	2	0.5
	CNM-CM-2846	Ear swab	16	8	1
	CNM-CM-4183	Bronchial aspirate	8	16	0.5
Geometric mean			6.96	6.06	1.15
S. apiospermum					
Geometric mean			6.21	2.62	0.73
Range			0.25–32	0.25–16	0.125–16

counting spores in a hematocytometer (1, 7, 9). *Aspergillus fumigatus* ATCC 2004305 and *Aspergillus flavus* ATCC 2004304 were used as quality control strains (6).

The antifungal agents used in the study were amphotericin B (range, 16 to 0.03 mg/liter) (Sigma Aldrich Química), itraconazole (range, 8 to 0.015 mg/liter) (Janssen S.A., Madrid, Spain), and voriconazole (range, 8 to 0.015 mg/liter) (Pfizer S.A.). The endpoint was the antifungal concentration that produced a complete inhibition of visual growth at 48 h.

Morphological identification. All strains were identified as *Scedosporium apiospermum* by conventional methods (3). We were not able to identify by morphological characteristics the new proposed species (4, 8).

Molecular identification. Figure 1 shows the phylogenetic analysis by means of maximum parsimony of a subset of the strains included in the study. Among 46 clinical strains analyzed, four *S. aurantiacum* strains were identified with a bootstrap value of ≥99. They were isolated from sputum, bronchial aspirate, a corneal sample, and an ear swab. In this collection of clinical isolates, we did not found any strain of *P. minutispora* or *P. angusta*, but the sequences obtained from GenBank were supported by bootstrap values of 73 and 65, respectively. On the other hand, the analyses of ITS sequences indicate that *Pseudallescheria ellipsoidea* and *P. fusoidea* are indistinguishable from *S. apiospermum* isolates (Fig. 1).

Antifungal susceptibility testing. The MICs of antifungal agents for the collection of clinical isolates are shown in Table 2. They are sorted by species identification by means of ITS sequencing.

It has always been suspected that *Pseudallescheria boydii* was a complex of species. Hitherto, morphological analyses of *Pseudallescheria boydii* isolates did not allow for a clear separation among different species. However, the analysis of DNA sequences has permitted the proposal of new species from *Pseudallescheria*, such as *S. aurantiacum*, *P. minutispora*, *P. angusta*, *P. ellipsoidea*, and *P. fusoidea* (4, 8).

We examined the prevalence of these new species in a collection of clinical isolates. ITS sequence analyses revealed the presence of four *S. aurantiacum* strains, obtained from an ocular sample, an ear swab, bronchial aspirate, and sputum, and no strains of *P. minutispora*. Although Gilgado et al. (4) have defined morphological characteristics to identify *S. aurantiacum* and *P. minutispora*, great experience in classical taxonomy is required to perform this task. Therefore, it is not expected that many clinical microbiology laboratories are able to identify those new species unless they use molecular methodology. Regarding susceptibility to antifungal drugs, *S. aurantiacum* seems slightly more resistant than *S. apiospermum* to amphotericin B and itraconazole, although a higher number of isolates should be analyzed before any conclusion is drawn (Table 2).

Because little is know about the prevalence of these new species and therefore there are no studies regarding epidemiology, pattern of disease, risk factors, antifungal susceptibility testing, etc., we strongly recommend sending all strains of *Scedosporium* species involved in human infections to reference laboratories where those isolates can be properly identified to the species level and antifungal susceptibility testing can be performed. In this way, the importance of these new species can be ascertained. Meanwhile, and especially for clinical use, it would be better to maintain the use of the *S. apiospermum* name. From a practical point of view and for clinical microbiology laboratories, we suggest performing antifungal susceptibility testing rather than applying molecular methods for the identification of these species.

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3.4.2. Actividad de 35 combinaciones de antifúngicos frente a *Scedosporium* spp.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 2008, p. 1136–1139 Vol. 52, No. 3
0066-4804/08/$08.00+0 doi:10.1128/AAC.01160-07

In Vitro Activities of 35 Double Combinations of Antifungal Agents against *Scedosporium apiospermum* and *Scedosporium prolificans*▽

Manuel Cuenca-Estrella,* Ana Alastruey-Izquierdo, Laura Alcazar-Fuoli, Leticia Bernal-Martinez, Alicia Gomez-Lopez, Maria J. Buitrago, Emilia Mellado, and Juan L. Rodriguez-Tudela

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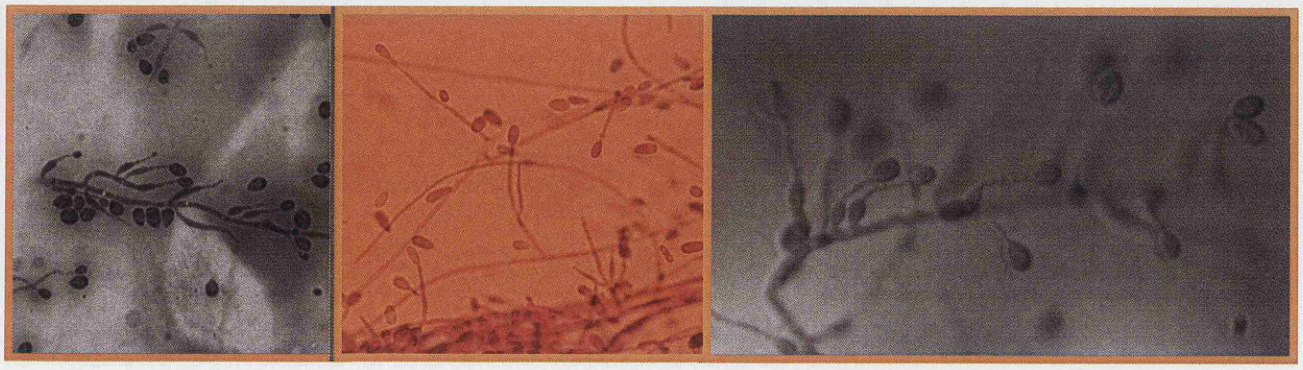
Received 3 September 2007/Returned for modification 3 November 2007/Accepted 29 December 2007

Resumen: Se ensayó la actividad de 35 combinaciones de antifúngicos frente a *Scedosporium* spp. mediante el método del "tablero de ajedrez" analizándose la concentración inhibitoria fraccionaria (CIF). En general las combinaciones tuvieron un efecto indiferente menos en las combinaciones de azoles y equinocandinas sobre *Scedosporium apiospermum*. No se detectó antagonismo para ninguna combinación en ninguna de las dos especies.



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Activities of 35 combinations of antifungal agents against *Scedosporium* spp. were analyzed by a checkerboard microdilution design and the summation of fractional concentration index. An average indifferent effect was detected apart from combinations of azole agents and echinocandins against *Scedosporium apiospermum*. Antagonism was absent for all antifungal combinations against both species.

Scedosporium apiospermum (*Pseudallescheria boydii*) is considered to be susceptible to voriconazole, posaconazole, and miconazole and appears to have various susceptibilities to itraconazole, ketoconazole, and amphotericin B. *S. prolificans* seems to be more resistant than *S. apiospermum* to antifungals, tolerating virtually all systemically active antifungal agents, including the new triazoles and echinocandins (2, 4, 6–10, 16, 17, 22).

Combination therapy could be an alternative to monotherapy for patients with invasive infections that are difficult to treat, such as those due to multiresistant species, and for those who fail to respond to standard treatment (5, 13, 14).

We have analyzed the in vitro activities of 35 combinations of broad-spectrum antifungal agents against a panel of clinical isolates of *S. apiospermum* and *S. prolificans*.

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Fungi. Twelve *S. apiospermum* clinical strains and 12 *S. pro-*

TABLE 1. Susceptibility results of 24 clinical isolates of *Scedosporium* spp.

Antifungal agent	MIC or MEC (μg/ml)[a]									
	Scedosporium apiospermum					*Scedosporium prolificans*				
	Range	Mode[b]	Geometric mean	50%	90%	Range	Mode	Geometric mean	50%	90%
Amphotericin B	0.25–>16.0	4.0	4.87	4.0	16.0	2.0–>16.0	16.0	13.9	16.0	>16.0
Flucytosine	>64.0	>64.0	>64.0	>64.0	>64.0	>64.0	>64.0	>64.0	>64.0	>64.0
Itraconazole	0.25–>8.0	8.0	3.28	4.0	>8.0	2.0–>8.0	8.0	>8.0	8.0	>8.0
Voriconazole	0.12–>8.0	1.0	0.93	1.0	4.0	1.0–>8.0	8.0	>8.0	>8.0	>8.0
Ravuconazole	0.50–>8.0	4.0	3.92	4.0	>8.0	2.0–>8.0	>8.0	>8.0	>8.0	>8.0
Posaconazole	0.12–>8.0	1.0	1.18	1.0	>8.0	1.0–>8.0	>8.0	>8.0	>8.0	>8.0
Terbinafine	4.0–>16.0	16.0	>16.0	16.0	>16.0	8.0–>16.0	16.0	>16.0	16.0	>16.0
Caspofungin	>16.0	>16.0	>16.0	>16.0	>16.0	>16.0	>16.0	>16.0	>16.0	>16.0
Caspofungin[c]	0.03–>16.0	8.0	2.56	0.50	>16.0	1.0–>16.0	1.0	6.56	8.0	>16.0
Micafungin	>16.0	>16.0	>16.0	>16.0	>16.0	>16.0	>16.0	>16.0	>16.0	>16.0
Micafungin[c]	0.03–>16.0	0.03	0.39	0.12	>16.0	0.03–>16.0	>16.0	2.69	8.0	>16.0
Anidulafungin	>16.0	>16.0	>16.0	>16.0	>16.0	>16.0	>16.0	>16.0	>16.0	>16.0
Anidulafungin[c]	0.50–4.0	1.0	1.19	1.0	4.0	1.0–4.0	4.0	4.0	4.0	4.0

[a] The table displays average results of two repetitions performed on different days.
[b] Mode, most frequent MIC value.
[c] MEC values are shown in this row.

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TABLE 2. Average FICi by *Scedosporium* spp. per antifungal combination[a]

Combination[b]	*Scedosporium apiospermum*				*Scedosporium prolificans*			
	MIC		MEC		MIC		MEC	
	FICi	No. (%) of strains showing synergy	FICi	No. (%) of strains showing synergy	FICi	No. (%) of strains showing synergy	FICi	No. (%) of strains showing synergy
AMB plus 5-FC	1.80	0/12 (0)	NC[c]	NC	2.0	0/12 (0)	NC	NC
AMB plus TBF	2.0	0/12 (0)	NC	NC	2.0	0/12 (0)	NC	NC
AMB plus ITC	0.93	1/12 (8.3)	NC	NC	2.0	0/12 (0)	NC	NC
AMB plus VRC	0.68	2/12 (16.6)	NC	NC	1.14	2/12 (16.6)	NC	NC
AMB plus POS	0.79	2/12 (16.6)	NC	NC	1.89	0/12 (0)	NC	NC
AMB plus RVC	0.82	3/12 (25)	NC	NC	1.51	0/12 (0)	NC	NC
AMB plus CPF	1.26	2/12 (16.6)	0.68	1/12 (8.3)	2.0	0/12 (0)	0.89	2/12 (16.6)
AMB plus MCF	1.87	0/12 (0)	0.85	4/12 (33.3)	2.0	0/12 (0)	1.55	0/12 (0)
AMB plus ADF	1.52	0/12 (0)	1.30	0/12 (0)	2.0	0/12 (0)	1.02	0/12 (0)
5-FC plus ITC	1.84	1/12 (8.3)	NC	NC	2.0	0/12 (0)	NC	NC
5-FC plus VRC	1.83	1/12 (8.3)	NC	NC	2.0	0/12 (0)	NC	NC
5-FC plus POS	1.86	0/12 (0)	NC	NC	2.0	0/12 (0)	NC	NC
5-FC plus RVC	1.84	1/12 (8.3)	NC	NC	2.0	0/12 (0)	NC	NC
5-FC plus CPF	2.0	0/12 (0)	2.0	0/12 (0)	2.0	0/12 (0)	2.0	0/12 (0)
5-FC plus MCF	2.0	0/12 (0)	2.0	0/12 (0)	2.0	0/12 (0)	2.0	0/12 (0)
5-FC plus ADF	2.0	0/12 (0)	1.87	0/12 (0)	2.0	0/12 (0)	1.88	0/12 (0)
TBF plus ITC	0.66	4/12 (33.3)	NC	NC	2.0	0/12 (0)	NC	NC
TBF plus VRC	1.44	0/12 (0)	NC	NC	1.13	3/12 (25)	NC	NC
TBF plus POS	1.09	0/12 (0)	NC	NC	1.91	0/12 (0)	NC	NC
TBF plus RVC	0.89	1/12 (8.3)	NC	NC	1.01	3/12 (25)	NC	NC
TBF plus CPF	1.29	1/12 (8.3)	1.57	1/12 (8.3)	1.38	0/12 (0)	0.65	1/12 (8.3)
TBF plus MCF	1.89	0/12 (0)	0.99	5/12 (41.7)	2.0	0/12 (0)	1.85	1/12 (8.3)
TBF plus ADF	1.81	0/12 (0)	1.91	0/12 (0)	2.0	0/12 (0)	1.77	1/12 (8.3)
ITC plus CPF	0.24	12/12 (100)	0.29	12/12 (100)	2.0	0/12 (0)	1.32	0/12 (0)
ITC plus MCF	0.54	6/12 (50)	0.24	8/12 (66.6)	2.0	0/12 (0)	2.0	0/12 (0)
ITC plus ADF	1.44	0/12 (0)	0.54	4/12 (33.3)	1.91	0/12 (0)	2.0	0/12 (0)
VRC plus CPF	0.62	3/12 (25)	0.41	8/12 (66.6)	2.0	0/12 (0)	1.41	2/12 (16.6)
VRC plus MCF	0.87	1/12 (8.3)	0.36	7/12 (58.3)	2.0	0/12 (0)	1.56	0/12 (0)
VRC plus ADF	1.73	0/12 (0)	0.66	0/12 (0)	2.0	0/12 (0)	1.14	1/12 (8.3)
POS plus CPF	0.39	8/12 (66.6)	0.39	9/12 (75)	2.0	0/12 (0)	1.70	0/12 (0)
POS plus MCF	0.92	5/12 (41.7)	0.39	6/12 (50)	2.0	0/12 (0)	1.89	0/12 (0)
POS plus ADF	1.73	0/12 (0)	0.66	2/12 (16.6)	2.0	0/12 (0)	1.79	0/12 (0)
RVC plus CPF	0.35	11/12 (91.7)	0.34	10/12 (83.4)	2.0	0/12 (0)	0.75	5/12 (41.7)
RVC plus MCF	0.38	7/12 (58.3)	0.28	11/12 (91.7)	2.0	0/12 (0)	2.0	0/12 (0)
RVC plus ADF	0.47	7/12 (58.3)	0.44	6/12 (50)	2.0	0/12 (0)	1.63	0/12 (0)

[a] The table shows results after two repetitions performed on different days.
[b] AMB, amphotericin B; 5-FC, flucytosine; TBF, terbinafine; ITC, itraconazole; VRC, voriconazole; POS, posaconazole; RVC, ravuconazole; CPF, caspofungin; MCF, micafungin; ADF, anidulafungin.
[c] NC, not calculated.

lificans strains were tested. The majority of isolates (n = 16) were obtained from blood cultures and the remainder (n = 8) from specimens of deep sites. *Aspergillus fumigatus* ATCC 204305 and *Aspergillus flavus* ATCC 204304 were included as quality control organisms (9, 20).

Antifungal agents. Antifungal agents used were amphotericin B (range, 16.0 to 0.03 μg/ml; Sigma-Aldrich Quimica S.A., Madrid, Spain), flucytosine (range, 64.0 to 0.12 μg/ml; Sigma-Aldrich), itraconazole (range, 8.0 to 0.015 μg/ml; Janssen S.A., Madrid, Spain), voriconazole (range, 8.0 to 0.015 μg/ml; Pfizer S.A., Madrid, Spain), ravuconazole (range, 8.0 to 0.015 μg/ml; Bristol-Myers Squibb, Princeton, NJ), posaconazole (range, 8.0 to 0.015 μg/ml; Schering-Plough, Kenilworth, NJ), terbinafine (range, 16.0 to 0.03 μg/ml; Novartis, Basel, Switzerland), caspofungin (range, 16.0 to 0.03 μg/ml; Merck & Co., Inc., Rahway, NJ), micafungin (range, 16.0 to 0.03 μg/ml; Astellas Pharma, Inc., Tokyo, Japan), and anidulafungin (range, 16.0 to 0.03 μg/ml; Pfizer S.A.).

Antifungal susceptibility testing. The individual MICs were determined by following the recommendations of the European Subcommittee for Antifungal Susceptibility Testing of the European Committee for Antimicrobial Susceptibility Testing (AFST-EUCAST) (1, 11, 15, 21, 23).

For amphotericin B, flucytosine, and azole compounds, the MIC was defined as the lowest concentration of the antifungal agent that completely inhibited fungal growth. For echinocandins, two different visual determinations of the endpoint were performed: (i) complete inhibition of growth (MIC) and (ii)

the lowest drug concentration resulting in aberrant hyphal growth by examination with an inverted microscope, or the minimum effective concentration (MEC) (3, 24).

Interaction of drugs in vitro. Drug interaction was evaluated in a checkerboard microdilution design. The combined effects were analyzed by summation of the fractional concentration index (FICi). For combinations including echinocandin, the FICi was also calculated taking into account both the MIC and the MEC of the echinocandin. The interactions were defined as synergistic if the FICi was ≤0.5, as antagonistic if the FICi was >4, and indifferent (or no interaction) if the FICi was >0.5 but ≤4. Duplicate testing was performed on two separate days.

Analysis of data. Descriptive statistical analysis of MIC, MEC, and FICi values was done with the Statistical Package for the Social Sciences (SPSS, version 15.0; SPSS S.L., Madrid, Spain).

Results and discussion. Table 1 shows the susceptibility testing results of *Scedosporium* clinical strains.

Regarding interactions of antifungal agents, a summary of the combined effects in vitro is displayed in Table 2. For *S. apiospermum*, an average indifferent effect was detected for combinations including amphotericin B, flucytosine, and terbinafine. However, average synergy was detected for some isolates and combinations of azole agents and echinocandins. In addition, some combinations showed synergy against a percentage of strains of *S. apiospermum*. Amphotericin B exhibited a positive effect against 15 to 25% of isolates when combined with azole agents. The combination of terbinafine plus itraconazole was synergistic against one-third of the isolates. The highest rates of synergy were obtained for azole agents plus echinocandins, particularly with itraconazole plus caspofungin, which exhibited synergy against 100% of strains. Other azole and echinocandin combinations also showed synergy against significant percentages of *S. apiospermum* isolates.

As for *Scedosporium prolificans*, all combinations were indifferent but synergy was detected for 15 to 25% of isolates for some combinations. The most active combination was ravuconazole plus caspofungin, which was synergistic against 41.7% of *S. prolificans* isolates when MEC values were used as endpoints.

Notably, antagonism was absent for all antifungal combinations against both *Scedosporium* spp.

The combined activity of antifungal agents against *Scedosporium* has rarely been evaluated previously. A report on the combined effect in vitro of amphotericin B and azole agents (miconazole, itraconazole, and fluconazole) against *S. apiospermum* was published in 1995 (26). Authors used the checkerboard technique and described an average indifferent effect, but synergy was found for some isolates, particularly for the amphotericin B and miconazole combination. Antagonism was not reported.

Meletiadis et al. reported that the combination of terbinafine with miconazole, voriconazole, or itraconazole showed synergy in vitro against *S. prolificans* (18, 19). The synergistic effects were more potent after 72 h of incubation. Authors used the checkerboard technique with three different reading methods, one spectrophotometrical and two colorimetric techniques. An alternative response surface approach method was used for assessing drug interaction as well (18).

Our results concur with those found years ago for *S. apiospermum*. Amphotericin B and azole agents showed synergy against a number of isolates, although an indifferent effect was the most common interaction. However, our data are somehow different from results reported by Meletiadis et al. We did not find a synergistic effect between terbinafine and azole agents in all cases. We observed synergy for 25% of *S. prolificans* isolates and only between voriconazole plus terbinafine and ravuconazole plus terbinafine.

We have obtained some novel results as well. Combinations including an azole agent plus an echinocandin exhibited synergy against most *S. apiospermum* isolates. The positive effect was particularly common for combinations including an azole compound plus caspofungin. Differences in combined activity of distinct echinocandins may be explained by different molecular interactions and limitations of the analysis by the FICi method. Further experiments are warranted in order to assess these interactions.

Data on the clinical efficacy of combination therapy in cases of *Scedosporium* infection are too scarce. Combinations of voriconazole plus terbinafine with or without aggressive surgical debridement have resulted in the cure or control of deep infections due to *S. prolificans* (12). In addition, a case of *S. prolificans* osteomyelitis was treated successfully with systemic administration of voriconazole and caspofungin (25).

There is insufficient evidence to make any recommendations for combination therapy, but azole agents plus echinocandins against *S. apiospermum* and terbinafine plus voriconazole against *S. prolificans* could have clinical efficacy.

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4. Discusión

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La epidemiología de las infecciones fúngicas está cambiando pues cada vez son más las especies que causan infecciones en humanos. Estas infecciones son más difíciles de diagnosticar y tratar ya que estos hongos emergentes son, en general, más resistentes a los antifúngicos disponibles por lo que están asociadas a una elevada mortalidad[126]. La correcta identificación de los hongos a nivel de especie y el estudio de su perfil de sensibilidad a los antifúngicos ayudan a la elección del tratamiento más adecuado, además de mejorar los datos epidemiológicos disponibles.

La identificación de los hongos filamentosos a nivel de especie mediante técnicas clásicas es una tarea lenta y difícil. Se basa principalmente en la observación de las características macro y microscópicas, y requiere de personal muy especializado. Aunque la observación morfológica de los hongos es insustituible, las técnicas moleculares ofrecen la ventaja de suplir al observador poco experimentado además de identificar especies indistinguibles morfológicamente entre sí o que no esporulan en cultivo, por lo que su empleo es cada vez más frecuente. Recientemente, un grupo de expertos ha acordado que la secuenciación de la región ITS del DNA ribosómico es la diana más apropiada para la identificación de hongos patógenos humanos[7,132]. Sin embargo, estas regiones ITSs no son suficientemente variables para distinguir entre especies próximas, como *A. lentulus* y *A. fumigatus*[8] o las especies del complejo *F. solani*[91]. En estos casos, es necesaria la utilización de otras dianas. El factor de elongación 1 α (EF-1α), la β tubulina o la calmodulina son algunos de los marcadores que se han utilizado, alternativamente a los ITSs, para la identificación de éstas especies.

El estudio del perfil de sensibilidad es también un factor importante a la hora de tratar las infecciones fúngicas. Se ha demostrado que las CMIs elevadas están asociadas con una respuesta clínica pobre[68,103]. Por ejemplo, *A. terreus* tiene CMIs más altas a anfotericina B que *A. fumigatus* hecho que ha sido asociado con una peor respuesta clínica a este fármaco en aquellos pacientes infectados con *A. terreus*[71,131]. El perfil de sensibilidad varía incluso en especies muy relacionadas, así *A. lentulus* es más resistente los azoles que *A. fumigatus*[8].

Por tanto la identificación de la especie causante de la infección y la determinación de su perfil de sensibilidad es imprescindible para la elección del tratamiento más correcto, sobretodo en infecciones invasoras donde el retraso del tratamiento o la utilización de un antifúngico poco activo tiene una relación directa con la supervivencia del paciente[18].

Durante el desarrollo de esta tesis se ha creado una base de datos de secuencias de ADN que actualmente contiene 5.700 entradas con el *software* InfoQuest (Biorad, España). Las secuencias de esta base de datos provienen tanto de cepas clínicas de la colección del Servicio de Micología, como de cepas tipo adquiridas en distintos centros de referencia (CBS, ATCC, CECT), además de secuencias de la base de datos del GenBank. En los casos en los que ha sido posible, las cepas han sido también identificadas mediante métodos morfológicos y se ha determinado su perfil de sensibilidad creándose otra base de datos con el paquete estadístico SPSS (Statistical Package for the Social Sciences). Los patógenos más frecuentes están representados con al menos 10 aislados por especie pero en la mayoría de los casos son muchos más. Todos las cepas tienen secuenciada la región ITS. Además, para géneros como *Aspergillus*, *Fusarium* y *Scedosporium* otros fragmentos como la β tubulina, el rodlet o el factor de elongación alfa también están disponibles.

Esta tesis ha estado principalmente enfocada en la identificación y el perfil de sensibilidad de los grupos de hongos filamentosos más frecuentemente aislados en infecciones humanas como son los géneros *Aspergillus, Fusarium* y *Scedosporium* y el orden mucorales. A continuación se discuten los resultados obtenidos en cada uno de los grupos.

4.1. *Aspergillus*

El género *Aspergillus* está dividido en siete subgéneros que a su vez se dividen en varias secciones. La región ITS es de utilidad para situar un aislado dentro de la sección a la que pertenece[7]. Sin embargo, no es lo suficientemente variable para la identificación de especies dentro algunas secciones, por lo que para ello se utilizan otras dianas como los genes que codifican para la β tubulina, el rodlet A o la calmodulina. El género *Aspergillus* es responsable de la inmensa mayoría de las infecciones fúngicas invasoras. Por esta razón es uno de los objetivos primarios de esta tesis. Dentro de este género se han analizado la sección *Fumigati*, la sección *Nigri* y la sección *Usti*.

4.1.1. Sección *Fumigati*

Las 28 cepas clínicas de *Aspergillus* previamente identificados como *A. fumigatus* atípicos, se clasificaron dentro de la sección *Fumigati* gracias a la secuenciación de la región ITS y sus características morfológicas. Mediante la secuenciación de una parte de los genes que codifican para la β tubulina y el rodlet A pudieron ser identificadas a nivel de especie. Catorce fueron identificados como *A. lentulus,* cinco como *Neosartorya hiratsukae*, cuatro como *Neosartorya pseudofischeri,* dos como *Aspergillus viridinutans,* dos como *Aspergillus fumigatiaffinis* y una como *Aspergillus fumisynnematus.* Por primera vez se describe a *A. fumisynnematus* y *A. fumigatiaffinis* como potencialmente patógenos en humanos.

A. fumigatus es generalmente sensible a los azoles y a la anfotericina B, aunque varios artículos describen cepas de *A. fumigatus* resistentes a los azoles[35,81,82]. Sin embargo, las especies de la sección *Fumigati* presentan diferentes patrones de sensibilidad a los antifúngicos. Todos los azoles, excepto el posaconazol, tenían CMIs elevadas frente a *A. lentulus, A. fumigatiaffinis, A. viridinutans* y *Neosartorya pseudofischeri.* La mayoría de las cepas de *A. lentulus* y *A. fumigatiaffinis* fueron también resistentes a la anfotericina B, mientras que *Neosartorya hiratsukae* y *Aspergillus fumisynnematus* fueron sensibles a todos los antifúngicos ensayados. La terbinafina mostró buena actividad frente a todas las especies del complejo *Fumigati.* Los escasos estudios previos realizados describen resultados similares[11,49,146]. Por el momento solo hay un estudio epidemiológico[9] realizado que informa de la prevalencia de estas especies en pacientes trasplantados de progenitores hematopoyéticos o de órgano sólido. En dicho estudio, se analizaron 218 cepas de *Aspergillus.* Mediante secuenciación de la región ITS se clasificaron 147 (67,4%) dentro de la sección *Fumigati* y la secuenciación de la β tubulina identificó a 139 (93,9 %) como *A. fumigatus,* y sólo un 6% como pertenecientes a otras especies de la sección, así cuatro fueron *A. lentulus* (2,7%), tres *N. udagawae* (2%) y uno *N. pseudofischeri* (0,7%).

Los resultados obtenidos indican que son necesarios más estudios epidemiológicos que pueden ayudar a descubrir cual es el papel que estas nuevas especies juegan en la infección fúngica invasora y si muchos de los fallos terapéuticos que se producen son debidos a infecciones causadas por especies resistentes como *A. lentulus.* Asimismo, es imprescindible la utilización de la identificación molecular (mediante secuenciación de fragmentos que codifican para la β tubulina, el rodlet A o la calmodulina) de aquellos *Aspergillus* aislados en casos probados y probables de infección fúngica invasora ya que dependiendo de la especie causante el tratamiento puede ser muy diferente.

4.1.2. Sección *Nigri*

La clasificación de un aislado de *Aspergillus* como perteneciente a la sección *Nigri* es fácil de realizar mediante métodos morfológicos. Sin embargo, mediante este trabajo[2] se quiso conocer cual era el perfil de sensibilidad de las especies de esta sección. Para ello 34 cepas de *Aspergillus* previamente identificados como *A. niger* por métodos morfológicos, se analizaron mediante la secuenciación de los ITS y una parte del gen que codifica para la β tubulina. Dieciocho fueron identificadas como *A. tubingensis*, 13 como *A. niger* y tres como *A. foetidus.*

En un trabajo previo en el que las cepas de *A. niger* no fueron identificadas molecularmente, se alcanzo la conclusión de que este hongo era sensible in vitro a voriconazol, posaconazol, anfotericina B y equinocandinas pero resistente a

itraconazol[22,23]. En este trabajo se demuestra que las cepas resistentes al itraconazol son *A. tubingensis* y *A. foetidus*, ya que la mayoría de los aislados de *A. niger* fueron sensibles a todos los antifúngicos. Esto pone de manifiesto la importancia de una correcta identificación a nivel de especie dentro de esta sección. En un estudio realizado en el Reino Unido[60] en el que se analizaron 43 aislados clínicos identificados mediante secuenciación de fragmentos de los genes que codifican para la calmodulina y la β tubulina, se encontraron patrones de sensibilidad comparables a los de este estudio, sin embargo la especie más frecuentemente aislada en este país fue *A. awamori* (58%) mientras que en las cepas analizadas de España no se encontró ningún aislado de esta especie, siendo la más frecuente *A. tubingensis* (53%) lo que implica diferencias epidemiológicas geográficas. Esto unido a las diferencias de sensibilidad pone de manifiesto la importancia de la identificación a nivel de especie mediante la secuenciación de otras dianas distintas a la región ITS para poder manejar las infecciones causadas por estos hongos de la manera más correcta posible.

4.1.3. Sección *Usti*

Debido a la aplicación de las técnicas moleculares, la taxonomía de la sección *Usti* ha cambiado recientemente. *A. calidoustus* ha sido descrita como la especie más comúnmente aislada en muestras clínicas. En el estudio realizado, se analizaron 9 cepas previamente identificadas como *A. ustus* según sus caracteres morfológicos, de las cuales 8 fueron identificadas como *A. calidoustus* y una como *A. pseudodeflectus*. En un artículo publicado previamente[138], tampoco se encontró ningún aislado clínico de *A. ustus*, por lo que probablemente las descripciones de casos clínicos humanos por *A. ustus*, corresponden a infecciones causadas por *A. calidoustus*. Asimismo, se identificó también una cepa de *A. pseudodeflectus*, especie que hasta ahora no había sido asociada con humanos.

El tratamiento de elección para estas infecciones no está establecido. Sin embargo, los datos obtenidos en este trabajo pueden ayudar a la elección del mismo ya que anfotericina B, terbinafina, micafungina y anidulafungina fueron los antifúngicos que tuvieron mejor actividad frente a las especies del grupo. Aunque el número de aislados analizado no permite alcanzar conclusiones, la información suministrada ayuda a la elección del antifúngico con la mejor actividad in vitro.

4.2. *Fusarium*

Las infecciones causadas por *Fusarium* han aumentado mucho en los últimos años[89] llegando a convertirse en uno de los grupos más importantes de hongos filamentosos en el ámbito clínico. En pacientes inmunocompetentes causan infecciones localizadas generalmente a causa de un trauma previo. Estas infecciones se resuelven normalmente con tratamiento antifúngico y cirugía. Sin embargo, en individuos inmunodeprimidos causan infecciones diseminadas con tasas de mortalidad del 50% al 90%, llegando prácticamente al 100 % cuando hay diseminación hematógena[36]. Esta alta tasa de mortalidad se debe en parte a que las especies de *Fusarium* son generalmente resistentes a todos los antifúngicos disponibles[5,40,99]. *F. solani* y *F. oxysporum* son las especies más comúnmente aisladas en muestras clínicas, sin embargo *F. verticilloides, Fusarium chlamydosporum, Fusarium dimerum, Fusarium napiforme, Fusarium nygamai, F. proliferatum* y *Fusarium sacchari* también han sido descritos como causantes de infecciones humanas[37,52,53]. En este género, debido a su relativa homogeneidad y a la existencia de parálogos la secuenciación de la región ITS no permite la identificación al nivel de especie[91,92]. Los análisis filogenéticos empleando otros genes como el factor de elongación alfa, la β tubulina, la calmodulina o la RNA polimerasa II, han revelado la existencia de numerosas especies crípticas dentro de cada morfoespecie, denominándose a estos grupos de especies "complejos". *F. solani* representa un complejo formado por más de 45 especies de las cuales al menos 20 han sido asociadas con infecciones humanas[91,93,149]. De la misma forma, existen el complejo de especies de *F. oxysporum, F. incarnatum-equiseti, F. dimerum* y *F. chlamydosporum*. La secuenciación del factor de elongación alfa es actualmente la diana más comúnmente empleada en la identificación molecular del género *Fusarium* ya que ha demostrado buenos resultados en numerosos estudios[65,91,92]. Mediante la secuenciación de este gen se pudieron identificar 67 aislados de *Fusarium* procedentes de muestras clínicas, 22 *F. solani,* 14 *F. oxysporum,* 14 *F. proliferatum,* 13 *F. verticillioides,* tres *F. equiseti* y un *F. reticulatum.*

La terbinafina y los azoles mostraron poca o nula actividad frente a las especies de *Fusarium* analizadas, siendo la anfotericina B el antifúngico más activo. Voriconazol y posaconazol fueron activos frente a algunos aislados. Se han usado diversos antifúngicos para el tratamiento de estas infecciones dando lugar a resultados dispares, por tanto se desconoce cual es el tratamiento de elección. Un estudio realizado por Kontoyiannis et. al.[68] indica que la recuperación de la neutropenia es el factor más importante para que los pacientes con infecciones por *Fusarium* sobrevivan. Por tanto, parece razonable emplear el antifúngico con mayor actividad in vitro que aunque no sea capaz de erradicar el hongo, permita el control de la infección hasta que una vez recuperada la inmunidad, el huésped

sea capaz de eliminarlo. Dado que el perfil de sensibilidad varía incluso dentro de una misma especie, es importante realizar un estudio de sensibilidad a cada cepa involucrada en un posible caso de fusariosis. La identificación morfológica y molecular de las especies del género *Fusarium,* es una tarea difícil que hoy por hoy está restringida a laboratorios con mucha experiencia. Una alternativa para el resto de los laboratorios sería la identificación a nivel de género mediante técnicas morfológicas o secuenciación de los ITS. Cualquier aislado sospechoso de ser el causante de una infección diseminada debe ser identificado a nivel de especie y su perfil de sensibilidad determinado para aportar todas las posibles alternativas al tratamiento del paciente.

4.3. Mucorales

El número de infecciones causadas por hongos mucorales ha aumentado en los últimos años y su tasa de mortalidad se acerca al 100% en algunos grupos de pacientes[110].

La identificación mediante las técnicas clásicas es complicada. Por ejemplo, *Mucor* y *Rhizomucor* tienen pocas diferencias morfológicas entre sí por lo que se confunden fácilmente: Tanto es así, que la aplicación de métodos moleculares ha descubierto que *Rhizomucor variabilis* pertenece en realidad al género *Mucor* Además, aunque *Lichtheimia* y *Rhizopus* son fáciles de identificar a nivel de género, su caracterización al nivel de especie es difícil. Para complicar más la situación, algunas especies como *Apophysomyces* y *Saksenaea* no suelen esporular en cultivo lo que hace su identificación mediante técnicas morfológicas imposible. Kontoyiannis et al.[69] compararon la identificación obtenida mediante secuenciación con la morfológica y observaron más de un 20% de discordancia. Por tanto, la secuenciación de las dianas apropiadas debe ser considerada la técnica de referencia para la identificación de este grupo de hongos.

Las 77 cepas clínicas de mucorales analizadas fueron correctamente identificadas mediante la secuenciación de la región ITS, lo que demuestra que esta diana es apropiada para su identificación. Como ya se ha descrito previamente[139] el género *Rhizomucor* resultó ser polifilético, agrupándose *Rhizomucor variabilis* con las especies del género *Mucor.*

Con respecto a la actividad de los antifúngicos frente a los mucorales, hay que destacar que todas las especies del grupo fueron resistentes a voriconazol. El uso extensivo de este azol ha sido relacionado con el aumento en el número de infecciones causadas por mucorales. Este aumento ha sido particularmente evidente desde la implantación del voriconazol como tratamiento de elección de la aspergilosis invasora[63,69,79,128]. Sin embargo, el incremento también puede deberse a un mayor número de pacientes con factores de riesgo así como al progreso de las herramientas de diagnóstico. Como otros

autores ya habían señalado, la anfotericina B es el antifúngico con mayor actividad frente a todas las especies del grupo excepto *Cunninghamella y Apophysomyces*[44,58,94,148,150]. La terbinafina es activa frente a un buen número de especies excepto *R. oryzae, M. circinelloides* y *R. variabilis.* Por otro lado, *R. pusillus* y *L. corymbifera* son sensibles in vitro a itraconazol, hecho que se ha demostrado en modelos experimentales realizados con *L. corymbifera,* por lo que puede representar una alternativa de tratamiento en las infecciones causadas estas especies. El primer azol con actividad frente a la mayoría de las especies es posaconazol, con la única excepción de *C. bertholletiae.* La combinación de posaconazol y anfotericina B ha mostrado buenos resultados en pacientes en los que la anfotericina B en monoterapia había fallado[86,104].

En resumen, los mucorales son un grupo heterogéneo de hongos con un perfil de sensibilidad a los antifúngicos muy variable que pueden ser fácilmente identificados mediante secuenciación de la región ITS. El tratamiento de elección actualmente es la anfotericina B, sin embargo el bajo porcentaje de curaciones y los problemas de toxicidad frecuentes, subrayan la necesidad de otras alternativas, como por ejemplo el posaconazol o las terapias combinadas.

4.3.1. *Lichtheimia*

El género *Absidia* es el tercer género en importancia clínica dentro de los mucorales[115] siendo responsable del 5% de los casos de mucormicosis. La identificación morfológica de los aislados consideraba que una sola especie, *Absidia corymbifera,* tenía importancia clínica. La utilización de métodos moleculares, además de fisiológicos y morfológicos, propuso la creación de un nuevo género, *Mycocladus,* en el que incluyeron a las especies termotolerantes de *Absidia* entre las que se encuentra *A. corymbifera* que pasó a llamarse *Mycocladus corymbifer*[56]. Posteriormente, se descubrió que la cepa tipo de *Mycocladus* (*Mycocladus verticillatus*) era posiblemente un cultivo mixto, por lo que el nombre del género no era válido y se cambió por el de *Lichtheimia*[57], descrito en 1903 por Vuillemin[140] ya que las características en la descripción original sí son compatibles con este nuevo grupo de *Absidia.*

Analizando las cepas de *Lichtheimia corymbifera* recibidas en el Servicio de Micología del Centro Nacional de Microbiología, se observaron dos grupos cuyas secuencias de ITS diferían más de un 2%, pero que morfológicamente eran prácticamente indistinguibles. Por tanto se hizo un estudio en el que se analizaron 54 cepas de *Lichtheimia* que procedían tanto de muestras ambientales como de muestras clínicas. Además, se incluyeron todas las cepas tipo disponibles en el CBS Fungal biodiversity Centre. Mediante el análisis de caracteres morfológicos, fisiológicos (crecimiento a 7 temperaturas) y criterios moleculares

(secuenciación de las regiones ITS, D1-D2 y parte del gen que codifica para la actina), se encontraron cinco especies: *L. corymbifera* (syn. *Absidia corymbifera, Mycocladus corymbifer*), *Lichtheimia ramosa* (Syn. *Absidia ramosa, Mycocladus ramosus*), *Lichtheimia ornata* com. nov., *Lichtheimia hyalospora* (syn. *A.hyalospora, M. hyalosporus*) y una nueva especie *Lichtheimia sphaerocystis* sp. nov. Los aislados clínicos pertenecían a las tres primeras especies. Por el contrario, el análisis de las secuencias de las respectivas dianas distinguió siete especies filogenéticas[134]. Los test de apareamiento solo confirmaron cinco de ellas, por lo que las 2 restantes, al no cumplir el concepto biológico de especie, no fueron reconocidas como tales. El análisis de los caracteres morfológicos y fisiológicos permitió la realización de una clave dicotómica de identificación de estas cinco especies.

En análisis del perfil de sensibilidad de las especies con relevancia clínica reveló que *L. ramosa* tiene CMIs ligeramente superiores a *L. corymbifera.* Al igual que en el resto de las especies de mucorales, la anfotericina B fue el fármaco más activo, el posaconazol fue el azol con mejor actividad y el voriconazol fue inactivo frente a todos los aislados. A diferencia de los otros géneros de mucorales, el itraconazol tiene actividad frente a la mayoría de los aislados (Media geométrica (MG) de las CMIs = 0,80 mg/L) aunque fue donde se observaron mayores diferencias entre las dos especies más comunes en muestras clínicas, *L. corymbifera* con MG CMIs de 0,56 mg/L y *L. ramosa* MG CMIs = 1 mg/L. Las equinocandinas fueron inactivas. Sólo se identifico un aislado como *L. ornata* por lo que, a pesar de que mantiene el patrón de resistencia al voriconazol y de sensibilidad a la anfotericina B y el posaconazol, no se pueden sacar conclusiones definitivas y es necesario el análisis de un mayor número de aislados que clarifiquen la relevancia clínica de esta especie, así como su perfil de sensibilidad.

En resumen, la aplicación del concepto filogenético y biológico de especie[134] permitió la distinción de cinco especies dentro del género *Lichtheimia: L. corymbifera*, *L. ramosa*, *L. ornata* com. nov., *L. hyalospora* y *L. sphaerocystis* sp. nov, pero es necesario el análisis de un mayor número de cepas para poder sacar alguna conclusión respecto a su patrón de sensibilidad y su epidemiología. El análisis filogenético de tres loci (ITS, D1-D2 y actina) aclaro de forma inequívoca la clasificación taxonómica del grupo. Sin embargo, la secuenciación de la región ITS es suficiente para obtener una correcta identificación a nivel de especie.

4.4. *Scedosporium*

El género *Scedosporium* consta de dos especies con importancia clínica: *S. apiospermum* (y su forma sexual *Pseudallescheria boydii*) y *S. prolificans*. Estos hongos causan infecciones localizadas debidas a traumatismos accidentales así como infecciones

diseminadas en individuos inmunodeprimidos. La mortalidad asociada a la infección invasora es cercana al 100 %[13,21,50]. El tratamiento es especialmente difícil debido a su naturaleza multiresistente. La aplicación de las técnicas moleculares para la identificación de este género ha demostrado que *S. apiospermum/ P. boydii* es, en realidad, un complejo de especies[42,43]. La existencia de las nuevas especies de *Scedosporium* fue evaluada en una colección de 46 aislados clínicos, previamente identificados morfológicamente como *S. apiospermum* morfológicamente. Mediante la secuenciación de la región ITS, cuatro cepas fueron identificadas como *S. aurantiacum* no encontrándose ningún aislado de *P. minutispora*. El estudio de sensibilidad a los antifúngicos de *S. aurantiacum* reveló pocas diferencias con *S. apiospermum* ya que los aislados tenían CMIs elevadas a anfotericina B e itraconazol mientras que voriconazol fue el antifúngico más activo. *S. prolificans* es aún más resistente a los antifúngicos, con CMIs elevadas frente a todos los antifúngicos ensayados. El tratamiento de elección para estas infecciones consiste en una combinación de tratamiento antifúngico y cirugía, pero el mejor antifúngico, la dosis más correcta y la duración del tratamiento se desconocen. El uso de terapias combinadas ha dado buenos resultados in vitro, así la combinación de anfotericina B y azoles es más activa que la monoterapia en algunos aislados de *S. apiospermum*[80]. Al ensayar 35 combinaciones de antifúngicos frente a cepas de *Scedosporium* la combinación de azoles y equinocandinas fue sinérgica frente a *S. apiospermum*. Sin embargo, únicamente la combinación de terbinafina y azoles fue sinérgica frente algunos aislados (25%) de *S. prolificans*.

Posteriormente a la publicación del artículo en el que se analiza la existencia de las nuevas especies en una colección de aislados clínicos de *Scedosporium,* se redefinió el género mediante la secuenciación de la β tubulina. Se descubrió que *P. boydii* no es la forma sexual de *S. apiospermum* sino otra especie diferente cuya forma asexual se denominó *Scedosporium boydii*. Además se describió una nueva especie que se ha denominado *Scedosporium dehoogii*. Nuestra colección de aislados clínicos se volvió a analizar mediante la secuenciación de la β tubulina (resultados sin publicar) y se encontró que de las cepas previamente identificadas como *S. apiospermum/ P. boydii,* 25 eran *S. apiospermum*, 13 *P. boydii (S. boydii),* tres *Pseudallescheria ellipsoidea* y una *S. dehoogii.* El perfil de sensibilidad de estas especies no tiene diferencias significativas con el de *S. apiospermum,* pero debido al número limitado de aislados no se puede alcanzar ninguna conclusión.

Ya se ha mencionado que el tratamiento de elección para las infecciones causadas por *Scedosporium* se desconoce pero es importante resaltar que se tratan de hongos multiresistentes, sobretodo *S. prolificans.* Por el momento la mejor opción consiste en la combinación de cirugía y antifúngicos. La prevalencia de las nuevas especies de

Scedosporium es baja y su perfil de sensibilidad no tiene diferencias significativas con el de *S. apiospermum*. Por tanto, la realización de estudios epidemiológicos y de factores de riesgo que identifiquen la importancia clínica de estas nuevas especies, y el análisis del perfil de sensibilidad de un mayor número de cepas puede ayudar a establecer recomendaciones que mejoren el tratamiento de las infecciones causadas por este grupo de patógenos.

4.5. Resumen de los principales hallazgos

La identificación mediante comparación de secuencias es un método rápido, económico y da resultados robustos objetivos y comparables entre laboratorios de cualquier localización. La secuenciación de la región ITS ha sido establecida como la técnica de referencia para la identificación de hongos patógenos humanos, sin menoscabo de la utilización de otras dianas cuando se requiera una identificación más exacta de algunos grupos de patógenos[7,132]. En este trabajo se ha comprobado que la secuenciación de la región ITS es una herramienta eficaz para la identificación de la mayoría de las especies de mucorales y para situar un aislado de *Aspergillus, Fusarium* y *Scedosporium* dentro del complejo al que pertenece, pero sobretodo para abordar la identificación de un hongo, a priori, desconocido. Para llegar a nivel de especie en los géneros *Aspergillus, Fusarium* y *Scedosporium,* la secuenciación de genes codificantes aporta la variabilidad necesaria para separar entre especies muy próximas que son indistinguibles mediante la secuenciación de los ITS. Así la β tubulina es útil para la identificación de las especies de *Aspergillus* y *Scedosporium,* y el factor de elongación alfa para las especies de *Fusarium.*

Por otro lado la aplicación de los métodos moleculares está identificando cuales son, en realidad, las especies de hongos que causan patología en el humano.

Es relevante analizar la importancia práctica que estas nuevas tecnologías tienen para el laboratorio clínico. Así, el análisis de la muestra donde se aísla el hongo o la enfermedad subyacente del paciente, puede indicar la relevancia clínica de la misma. Es evidente que cuando se trata de muestras provenientes de lugares estériles (hemocultivos, líquido cefalorraquídeo, biopsias, etc.) o pacientes con alto riesgo de padecer una infección fúngica (transplantados, pacientes oncohematológicos, SIDA, etc.) la identificación a nivel de especie y el estudio del perfil de sensibilidad se convierten en herramientas imprescindibles para elegir el tratamiento más correcto y monitorizar la evolución de la infección, además de su intrínseco valor epidemiológico.

La identificación morfológica no puede ser sustituida por la identificación molecular ya que constituye una herramienta básica para confirmar la identificación obtenida por

comparación de secuencias, así como para descartar posibles contaminaciones en el proceso de identificación molecular.

Las bases de datos que normalmente se utilizan para identificar los hongos patógenos humanos contienen un numero excesivo de errores como para utilizarlas de forma rutinaria por lo que los resultados obtenidos deben analizarse con cautela. La creación de bases de datos con aislados perfectamente caracterizados, es un paso imprescindible que ya se está llevando a cabo.

El incremento de las infecciones fúngicas, la aparición de nuevos patógenos y el aumento en la disponibilidad del número de antifúngicos para tratar dichas infecciones hace que el estudio del perfil de sensibilidad sea una herramienta indispensable para un correcto manejo de la infección. Las especies implicadas en infecciones humanas tienen patrones de sensibilidad muy diferentes, siendo las especies emergentes las que presentan mayor porcentaje de cepas resistentes.

4.6. Aplicación de los resultados y transferencia de tecnología

Con la realización de esta tesis se ha desarrollado una base de datos de secuencias y de sensibilidad a los antifúngicos de los principales hongos patógenos humanos. La base de datos de secuencias contiene tanto cepas de referencia (cepas tipo) como cepas clínicas y ha sido apoyada con la identificación morfológica de todos los aislados. Esta base de datos representa por tanto una herramienta robusta y confiable y ya ha tenido una aplicación inmediata en diversos campos:

1. Actualmente se está utilizando para la identificación molecular de los hongos que llegan al Servicio de Micología del Centro Nacional de Microbiología procedentes del Sistema Nacional de Salud.

2. Esta base de datos ha servido para diseñar sondas específicas para los principales patógenos humanos que se han utilizado para desarrollar técnicas rápidas de identificación y diagnóstico de la infección fúngica basadas en la técnica de la PCR en tiempo real.

3. Asimismo, la base de datos de secuencias ha servido para desarrollar sondas específicas que han sido la base del incipiente desarrollo de un microarray para la identificación de los principales hongos patógenos humanos mediante la tecnología SSELO (sequence-specific end-labelling of the oligonucleotides) que se caracteriza por una alta especificidad (discriminación de 1 pb) y una alta sensibilidad de detección.

4. En colaboración con el CDC (Centers for Disease Control and Prevention) y el CBS (Fungal biodiversity Centre) se va a desarrollar una plataforma Web de identificación que aporte además datos de sensibilidad a los antifúngicos de las especies clínicas de mucorales.

5. Conclusiones

5. Conclusiones

1. La secuenciación de la región ITS es la herramienta más adecuada para realizar la identificación de un hongo desconocido aislado de una infección humana. Permite también la identificación a nivel de especie de los hongos del orden mucorales y la clasificación dentro de sección o complejo en los géneros *Aspergillus, Fusarium* y *Scedosporium.*

2. El estudio del perfil de sensibilidad a los antifúngicos es una herramienta imprescindible para dar el mejor tratamiento a una infección fúngica. La emergencia de especies resistentes subraya este hecho.

3. La sección *Fumigati* del género *Aspergillus* presenta patrones de sensibilidad distintos que son dependientes de especie. La identificación a nivel de especie mediante la secuenciación del gen que codifica para la β tubulina permite la clasificación de estos aislados dentro de uno de los patrones de resistencia existentes.

4. La identificación a nivel de especie de la sección *Nigri* del género *Aspergillus* se consigue mediante la secuenciación del gen que codifica para la β tubulina. Los diferentes patrones de resistencia de esta sección son dependientes de cepa, por lo que la realización de test de sensibilidad es la herramienta más adecuada para el manejo de estas infecciones.

5. Las especies de *Aspergillus* pertenecientes a la sección *Usti* se identifican a nivel de especie mediante la secuenciación del gen que codifica para la β tubulina. Aunque, el escaso número de cepas analizadas no permite sacar conclusiones claras respecto a la importancia de la determinación del perfil de sensibilidad, los datos obtenidos apuntan a que no hay grandes diferencias dentro de la sección, por lo que sería suficiente con la identificación a nivel de sección mediante la secuenciación de la región ITS.

6. Las distintas especies del género *Fusarium* y del grupo mucorales presentan diferencias de sensibilidad intraespecíficas. Por tanto es necesario la determinación del perfil de sensibilidad en todas aquellas cepas aisladas en infecciones probadas y probables para un correcto manejo de la infección.

7. Las especies del género *Scedosporium* son multiresistentes. El análisis del perfil de sensibilidad de las nuevas especies no reflejó diferencias significativas, aunque *Scedosporium aurantiacum* es ligeramente más resistente a los antifúngicos que *S.*

apiospermum. Sin embargo, es necesario el análisis de más aislados de las nuevas especies para poder dar recomendaciones en este sentido.

8. La combinación del concepto biológico y filogenético de especie permitió la clasificación taxonómica del género *Lichtheimia* quedando compuesto por cinco especies, *L. corymbifera, L. ramosa, L. hyalospora, L. ornata* y una nueva especie *Lichtheimia sphaerocystis.*

9. La base de datos desarrollada como consecuencia de este trabajo, representa una herramienta robusta y confiable que se está utilizando actualmente en el Servicio de Micología para la identificación mediante comparación de secuencias y para el desarrollo de diversas herramientas de identificación (PCR tiempo real, microarrays y plataforma Web de identificación y sensibilidad de mucorales).

Conclusions

1. The sequencing of the ITS region is the most suitable tool to achieve the correct identification of an unknown fungal strain. The ITS region allows the correct identification to species level in mucorales species and the classification into section or species complex in *Aspergillus, Fusarium* and *Scedosporium.*

2. The susceptibility profile is an essential tool for the correct management of fungal infections. This fact is highlighted by the increase of multiresistant species.

3. The species of *Aspergillus* section *Fumigati* have different susceptibility profiles. The identification to species level by means of β tubulin sequencing allows the classification of these isolates into one of the susceptibility profiles present.

4. The identification to species level of *Aspergillus* section *Nigri* can be done by means of sequencing part of the β tubulin gene. Different susceptibility profiles present in this section are isolate-dependant, therefore the susceptibility profile must be determined in order to provide the best treatment.

5. The species of *Aspergillus* section *Usti* are identified to species level by means of β tubulin sequencing. Although the small number of isolates analyzed does not allow to draw any clear conclusion in their susceptibility profile, the results showed that no big differences are present; therefore the identification to species complex level by ITS sequencing could be enough to achieve the best treatment.

6. The susceptibility profile of *Fusarium* spp. and mucorales species have is isolate dependent. Therefore the determination of susceptibility profile in any strain implicated in probable and proven infections is warranted.

7. *Scedosporium* species are multiresistant. The analysis of the susceptibility profile of the recently described new species yields no significant differences. However, *Scedosporium aurantiacum* is slightly more resistant to antifungals than *Scedosporium apiospermum.* The analysis of more strains to determine the real importance of this observation is warranted

8. The combination of Genealogical Concordance Phylogenetic Species Recognition and Biological Species Recognition allow us the taxonomical classification of *Lichtheimia* species. This genus currently contains five species *L. corymbifera, L. ramosa, L. ornata,*

L. hyalospora and a new specie: *Lichtheimia sphaerocystis A.* Alastruey-Izquierdo and G. Walther, sp. nov. Only the first three are clinically relevant.

9. The database developed with this work represents a trustable and robust tool for the identification of fungal human pathogens. This database is being used in the Mycology Reference Laboratory of Spain for the identification of clinical strains to species level by means of sequencing comparison and for the development of identification tools: (i) real time PCR, (ii) microarray technology and (iii) web tool for identification of mucorales.

6. Bibliografía

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7. Anexos

Anexo I.- Secuencias introducidas en la base de datos GenBank

Cepa	Especie	Número de acceso GenBank		
		β tubulina	rodlet A	Cyp51 A
CBS121601	*Aspergillus calidoustus*	FJ624456		
CNM-CM2105	*Aspergillus calidoustus*	FJ624457		
CNM-CM2272	*Aspergillus calidoustus*	FJ624458		
CNM-CM2475	*Aspergillus calidoustus*	FJ624460		
CNM-CM3788	*Aspergillus calidoustus*	FJ624461		
CNM-CM3927	*Aspergillus calidoustus*	FJ624462		
CNM-CM4115	*Aspergillus calidoustus*	FJ624465		
CNM-CM4212	*Aspergillus calidoustus*	FJ624466		
CNM-CM 4002	*Aspergillus foetidus*	FJ828909		
CNM-CM 4995	*Aspergillus foetidus*	FJ828922		
CNM-CM 5264	*Aspergillus foetidus*	FJ828925		
CNM-CM-2280	*Aspergillus fumigatiaffinis*	EU310841	EU310808	
CNM-CM-3227	*Aspergillus fumigatiaffinis*	EU310844	EU310811	
CNM-CM-3248	*Aspergillus fumigatus*	EU310845	EU310812	
CNM-CM-3254	*Aspergillus fumigatus*	EU310846	EU310813	
CNM-CM-3258	*Aspergillus fumigatus*	EU310847	EU310814	
CNM-CM-3652	*Aspergillus fumigatus*	EU310855	EU310822	
CNM-CM-3722	*Aspergillus fumigatus*	EU310856	EU310823	
CNM-CM 1244	*Aspergillus fumigatus*			EU626227
CNM-CM 796	*Aspergillus fumigatus*			EU626228
CNM-CM 2097	*Aspergillus fumigatus*			EU626229
CNM-CM 3500	*Aspergillus fumigatus*			EU626230
CNM-CM 1245	*Aspergillus fumigatus*			EU626231
CNM-CM 2159	*Aspergillus fumigatus*			EU626232
CNM-CM 2164	*Aspergillus fumigatus*			EU626233
CNM-CM 4593	*Aspergillus fumigatus*			EU626234
CNM-CM 2627	*Aspergillus fumigatus*			EU626235
CNM-CM 2733	*Aspergillus fumigatus*			EU626236
CNM-CM 3248	*Aspergillus fumigatus*			EU626237
CNM-CM237	*Aspergillus fumigatus*	FJ624459		
CNM-CM-4063	*Aspergillus fumisynnematus*	EU310862	EU310829	
CNM-CM-1290	*Aspergillus lentulus*	EU310839	EU310806	
CNM-CM-3134	*Aspergillus lentulus*	EU310842	EU310809	
CNM-CM-3364	*Aspergillus lentulus*	EU310850	EU310817	
CNM-CM-3537	*Aspergillus lentulus*	EU310851	EU310818	
CNM-CM-3538	*Aspergillus lentulus*	EU310852	EU310819	
CNM-CM-3583	*Aspergillus lentulus*	EU310853	EU310820	
CNM-CM-3599	*Aspergillus lentulus*	EU310854	EU310821	
CNM-CM-4330	*Aspergillus lentulus*	EU310864	EU310831	
CNM-CM-4370	*Aspergillus lentulus*	EU310865	EU310832	
CNM-CM-4387	*Aspergillus lentulus*	EU310866	EU310833	
CNM-CM-4415	*Aspergillus lentulus*	EU310867	EU310834	
CNM-CM-4420	*Aspergillus lentulus*	EU310868	EU310835	
CNM-CM-4426	*Aspergillus lentulus*	EU310869	EU310836	
CNM-CM-4428	*Aspergillus lentulus*	EU310870	EU310837	
CNM-CM 3236	*Aspergillus niger*	FJ828895		
CNM-CM 3257	*Aspergillus niger*	FJ828896		

Cepa	Especie	Número de acceso GenBank		
		β tubulina	rodlet A	Cyp51 A
CNM-CM 3586	*Aspergillus niger*	FJ828901		
CNM-CM 3636	*Aspergillus niger*	FJ828902		
CNM-CM 3641	*Aspergillus niger*	FJ828903		
CNM-CM 3672	*Aspergillus niger*	FJ828905		
CNM-CM 4004	*Aspergillus niger*	FJ828911		
CNM-CM 4213	*Aspergillus niger*	FJ828913		
CNM-CM 4262	*Aspergillus niger*	FJ828914		
CNM-CM 4316	*Aspergillus niger*	FJ828917		
CNM-CM 5095	*Aspergillus niger*	FJ82892		
CNM-CM5070	*Aspergillus pseudodeflectus*	FJ624467		
CNM-CM 3123	*Aspergillus tubingensis*	FJ828892		
CNM-CM 3125	*Aspergillus tubingensis*	FJ828893		
CNM-CM 3177	*Aspergillus tubingensis*	FJ828894		
CNM-CM 3507	*Aspergillus tubingensis*	FJ828898		
CNM-CM 3585	*Aspergillus tubingensis*	FJ828900		
CNM-CM 3654	*Aspergillus tubingensis*	FJ828904		
CNM-CM 3810	*Aspergillus tubingensis*	FJ828906		
CNM-CM 4000	*Aspergillus tubingensis*	FJ828907		
CNM-CM 4001	*Aspergillus tubingensis*	FJ828908		
CNM-CM 4003	*Aspergillus tubingensis*	FJ828910		
CNM-CM 4005	*Aspergillus tubingensis*	FJ828912		
CNM-CM 4264	*Aspergillus tubingensis*	FJ828915		
CNM-CM 4296	*Aspergillus tubingensis*	FJ828916		
CNM-CM 4352	*Aspergillus tubingensis*	FJ828918		
CNM-CM 4688	*Aspergillus tubingensis*	FJ828919		
CNM-CM 4897	*Aspergillus tubingensis*	FJ828920		
CNM-CM 4899	*Aspergillus tubingensis*	FJ828921		
CNM-CM 5094	*Aspergillus tubingensis*	FJ828923		
CNM-CM4036	*Aspergillus ustus*	FJ624463		
CNM-CM4037	*Aspergillus ustus*	FJ624464		
CNM-CM-3147	*Aspergillus viridinutans*	EU310843	EU310810	
CNM-CM-4518	*Aspergillus viridinutans*	EU310871	EU310838	
CNM-CM-3303	*Neosartorya hiratsukae*	EU310848	EU310815	
CNM-CM-3305	*Neosartorya hiratsukae*	EU310849	EU310816	
CNM-CM-3740	*Neosartorya hiratsukae*	EU310857	EU310824	
CNM-CM-3764	*Neosartorya hiratsukae*	EU310858	EU310825	
CNM-CM-4328	*Neosartorya hiratsukae*	EU310863	EU310830	
CNM-CM-2270	*Neosartorya pseudofischeri*	EU310840	EU310807	
CNM-CM-3769	*Neosartorya pseudofischeri*	EU310859	EU310826	
CNM-CM-3914	*Neosartorya pseudofischeri*	EU310860	EU310827	
CNM-CM-4060	*Neosartorya pseudofischeri*	EU310861	EU310828	

Cepa	Especie	Número de acceso GenBank			
		ITS	LSU	Actina	
				paralog I	paralog II
CBS 100.17	*Lichtheimia corymbifera*_c2			GQ342820	
CBS 100.17	*Lichtheimia corymbifera*_c4	GQ342885	GQ342942		GQ342715
CBS 100.51	*Lichtheimia corymbifera*_c2	GQ342886	GQ342939	GQ342826	
CBS 100.51	*Lichtheimia corymbifera*_c6				GQ342719
CBS 100.51	*Lichtheimia corymbifera*_c7			GQ342824	
CBS 102.48	*Lichtheimia corymbifera*_c4	GQ342888	GQ342910		GQ342718
CBS 102.48	*Lichtheimia corymbifera*_c5			GQ342821	
CBS 115811	*Lichtheimia corymbifera*_c4	GQ342887	GQ342932		GQ342720
CBS 115811	*Lichtheimia corymbifera*_c6			GQ342833	
CBS 115811	*Lichtheimia corymbifera*_c8			GQ342829	
CBS 100.31	*Lichtheimia corymbifera* _c2	GQ342879	GQ342914		GQ342722
CBS 100.31	*Lichtheimia corymbifera* _c3				GQ342714
CBS 100.31	*Lichtheimia corymbifera* _c5			GQ342825	
CBS 100.31	*Lichtheimia corymbifera* _c8			GQ342822	
CBS 101040	*Lichtheimia corymbifera* _c1	GQ342882	GQ342918		GQ342723
CBS 101040	*Lichtheimia corymbifera* _c4				GQ342721
CBS 101040	*Lichtheimia corymbifera* _c7			GQ342830	
CBS 101040	*Lichtheimia corymbifera* _c8			GQ342819	
CBS 109940	*Lichtheimia corymbifera* _c1	GQ342881	GQ342917		
CBS 109940	*Lichtheimia corymbifera* _c4			GQ342817	
CBS 120580	*Lichtheimia corymbifera* _c3	GQ342884	GQ342919		GQ342713
CBS 120580	*Lichtheimia corymbifera* _c8			GQ342828	
CBS 120581	*Lichtheimia corymbifera* _c3	GQ342883	GQ342948		
CBS 120581	*Lichtheimia corymbifera* _c4				GQ342716
CBS 120581	*Lichtheimia corymbifera* _c8			GQ342823	
CBS 120805	*Lichtheimia corymbifera* _c3	GQ342880	GQ342915	GQ342818	
CBS 120805	*Lichtheimia corymbifera* _c4				GQ342717
CBS 429.75	*Lichtheimia corymbifera*_c1	GQ342878	GQ342903		GQ342712
CBS 429.75	*Lichtheimia corymbifera*_c6			GQ342831	
CBS 519.71	*Lichtheimia corymbifera*_c4	GQ342889	GQ342904		
CBS 519.71	*Lichtheimia corymbifera*_c6			GQ342827	
CBS 519.71	*Lichtheimia corymbifera*_c7			GQ342832	
CBS 100.36	*Lichtheimia hyalospora*_c1	GQ342898	GQ342943	GQ342750	
CBS 100.36	*Lichtheimia hyalospora*_c3	GQ342897		GQ342751	
CBS 173.67	*Lichtheimia hyalospora*_c2	GQ342893	GQ342905	GQ342755	
CBS 102.36	*Lichtheimia hyalospora*_c3	GQ342895	GQ342907	GQ342752	
CBS 102.36	*Lichtheimia hyalospora*_c4			GQ342753	
CBS 518.71	*Lichtheimia hyalospora*_c3			GQ342756	
CBS 518.71	*Lichtheimia hyalospora*_c5	GQ342894	GQ342944	GQ342754	
CBS 100.28	*Lichtheimia hyalospora*_c3		GQ342902	GQ342748	
CBS 100.28	*Lichtheimia hyalospora*_c4	GQ342896	GQ342950	GQ342749	
CNM-CM4978	*Lichtheimia ornata*_c2	GQ342892			GQ342727
CNM-CM4978	*Lichtheimia ornata*_c8			GQ342835	
CBS 958.68	*Lichtheimia ornata* _c2	GQ342890	GQ342936		GQ342726
CBS 958.68	*Lichtheimia ornata* _c4			GQ342834	
CBS 291.66	*Lichtheimia ornata*_c2	GQ342891	GQ342946		GQ342724
CBS 291.66	*Lichtheimia ornata*_c3				GQ342725

Cepa	Especie	Número de acceso GenBank			
		ITS	LSU	Actina	
				paralog I	paralog II
CBS 291.66	*Lichtheimia ornata_c7*			GQ342836	
CBS 291.66	*Lichtheimia ornata_c8*			GQ342837	
CBS 100.24	*Lichtheimia ramosa_c3*	GQ342876	GQ342941	GQ342814	
CBS 100.24	*Lichtheimia ramosa_c4*			GQ342804	
CBS 100.49	*Lichtheimia ramosa_c2*	GQ342858	GQ342940	GQ342812	
CBS 100.49	*Lichtheimia ramosa_c3*			GQ342791	
CBS 100.55	*Lichtheimia ramosa_c3*			GQ342771	
CBS 100.55	*Lichtheimia ramosa_c3b*	GQ342851	GQ342938	GQ342766	
CBS 101.51	*Lichtheimia ramosa_c4*	GQ342859	GQ342945		GQ342747
CBS 101.51	*Lichtheimia ramosa_c5*			GQ342796	
CBS 101.51	*Lichtheimia ramosa_c7*			GQ342789	
CBS 101.55	*Lichtheimia ramosa_c1*	GQ342865	GQ342947	GQ342788	
CBS 101.55	*Lichtheimia ramosa_c2*				GQ342731
CBS 124198	*Lichtheimia ramosa_c2*	GQ342848	GQ342906		GQ342838
CBS 124198	*Lichtheimia ramosa_c6*			GQ342841	
CBS 124198	*Lichtheimia ramosa_c7*			GQ342840	
CBS 223.78	*Lichtheimia ramosa_c7*	GQ342877	GQ342934	GQ342807	
CBS 223.78	*Lichtheimia ramosa_ss2_c2*				GQ342734
CBS 223.78	*Lichtheimia ramosa_ss2_c6*				GQ342739
CBS 223.78	*Lichtheimia ramosa_ss2_c7*			GQ342811	
CBS 271.65	*Lichtheimia ramosa_c2*	GQ342875	GQ342937		GQ342746
CBS 271.65	*Lichtheimia ramosa_c3*				GQ342740
CBS 271.65	*Lichtheimia ramosa_c7*			GQ342805	
CBS 271.65	*Lichtheimia ramosa_c8*			GQ342816	
CBS 649.78	*Lichtheimia ramosa_c1*	GQ342849	GQ342912		GQ342728
CBS 649.78	*Lichtheimia ramosa_c3*			GQ342779	
CBS 649.78	*Lichtheimia ramosa_c4*			GQ342781	
CBS 713.74	*Lichtheimia ramosa_c1*	GQ342856	GQ342935	GQ342797	
CBS 713.74	*Lichtheimia ramosa_c4*				GQ342737
CNM-CM2166	*Lichtheimia ramosa_c2*	GQ342863	GQ342926	GQ342798	
CNM-CM2166	*Lichtheimia ramosa_c4*			GQ342792	
CNM-CM3590	*Lichtheimia ramosa_c2*				GQ342741
CNM-CM3590	*Lichtheimia ramosa_c4*			GQ342786	
CNM-CM3590	*Lichtheimia ramosa_c6*			GQ342810	
CNM-CM3590	*Lichtheimia ramosa_c8*	GQ342869	GQ342924	GQ342785	
CNM-CM3590	*Lichtheimia ramosa_c8b*				GQ342744
CNM-CM4119	*Lichtheimia ramosa_c1*	GQ342862	GQ342923		GQ342742
CNM-CM4119	*Lichtheimia ramosa_c2*			GQ342793	
CNM-CM4119	*Lichtheimia ramosa_c4*			GQ342803	
CNM-CM4228	*Lichtheimia ramosa_c1*	GQ342861	GQ342922	GQ342787	
CNM-CM4228	*Lichtheimia ramosa_c2*			GQ342794	
CNM-CM4228	*Lichtheimia ramosa_c3*				GQ342729
CNM-CM4253	*Lichtheimia ramosa_c4*	GQ342860	GQ342921	GQ342795	
CNM-CM4253	*Lichtheimia ramosa_c6*				GQ342733
CNM-CM4253	*Lichtheimia ramosa_c7*			GQ342780	
CNM-CM4261	*Lichtheimia ramosa_c1*			GQ342776	
CNM-CM4261	*Lichtheimia ramosa_c2*	GQ342854	GQ342953	GQ342767	
CNM-CM4337	*Lichtheimia ramosa_c4*	GQ342852	GQ342920	GQ342765	
CNM-CM4427	*Lichtheimia ramosa_c5*	GQ342853	GQ342931	GQ342773	

Cepa	Especie	Número de acceso GenBank			
		ITS	LSU	Actina	
				paralog I	paralog II
CNM-CM4537	*Lichtheimia ramosa*_c4	GQ342873	GQ342930	GQ342772	
CNM-CM4537	*Lichtheimia ramosa*_c6			GQ342777	
CNM-CM4849	*Lichtheimia ramosa*_c1	GQ342855	GQ342929	GQ342769	
CNM-CM4849	*Lichtheimia ramosa*_c2	GQ342868	GQ342952	GQ342778	
CNM-CM5111	*Lichtheimia ramosa*_c1	GQ342871	GQ342928		GQ342735
CNM-CM5111	*Lichtheimia ramosa*_c3				GQ342743
CNM-CM5111	*Lichtheimia ramosa*_c8			GQ342806	
CNM-CM5111	*Lichtheimia ramosa*_ss1_c2			GQ342783	
CNM-CM5111	*Lichtheimia ramosa*_ss1_c5			GQ342784	
CNM-CM5111	*Lichtheimia ramosa*_ss1_c6			GQ342808	
CNM-CM5111	*Lichtheimia ramosa*_ss1_c7				GQ342736
CNM-CM5171	*Lichtheimia ramosa*_c2	GQ342864	GQ342927		GQ342732
CNM-CM5171	*Lichtheimia ramosa*_c5			GQ342790	
CNM-CM5171	*Lichtheimia ramosa*_c6			GQ342799	
CBS 112528	*Lichtheimia ramosa* _c1	GQ342850	GQ342913	GQ342813	
CBS 112528	*Lichtheimia ramosa* _c3			GQ342764	
CBS 124197	*Lichtheimia ramosa* _c1	GQ342870	GQ342951	GQ342845	
CBS 124197	*Lichtheimia ramosa* _c5			GQ342846	
CBS 124197	*Lichtheimia ramosa* _c6			GQ342842	
CBS 124197	*Lichtheimia ramosa* _ss1_c1			GQ342843	
CBS 124197	*Lichtheimia ramosa* _ss1_c3			GQ342844	
CBS 124197	*Lichtheimia ramosa* _ss1_c7				GQ342839
CBS 269.65	*Lichtheimia ramosa* _c3	GQ342857	GQ342949		GQ342738
CBS 269.65	*Lichtheimia ramosa* _c6			GQ342802	
CBS 269.65	*Lichtheimia ramosa* _c7			GQ342801	
CNM-CM1638	*Lichtheimia ramosa* _c2	GQ342866	GQ342954	GQ342800	
CNM-CM1638	*Lichtheimia ramosa* _c3				GQ342730
CNM-CM3148	*Lichtheimia ramosa* _c4			GQ342782	
CNM-CM3148	*Lichtheimia ramosa* _c6			GQ342775	
CNM-CM3148	*Lichtheimia ramosa* _c8	GQ342872	GQ342925	GQ342768	
AS 3.4808	*Lichtheimia ramosa*_c1	GQ342867	GQ342955	GQ342770	
AS 3.4808	*Lichtheimia ramosa*_c6			GQ342774	
CBS 582.65	*Lichtheimia ramosa*_c1			GQ342809	
CBS 582.65	*Lichtheimia ramosa*_c2	GQ342874	GQ342909		GQ342745
CBS 582.65	*Lichtheimia ramosa*_c5			GQ342815	
CBS 103.35	*Lichtheimia ramosa*_c2	GQ342847	GQ342908	GQ342763	
CBS 420.70	*Lichtheimia sphaerocystis*_c1			GQ342761	
CBS 420.70	*Lichtheimia sphaerocystis* _c4	GQ342900	GQ342933	GQ342760	
CBS 647.78	*Lichtheimia sphaerocystis* _c1	GQ342899	GQ342911	GQ342757	
CBS 647.78	*Lichtheimia sphaerocystis* _c3			GQ342759	
CBS 648.78	*Lichtheimia sphaerocystis* _c3	GQ342901	GQ342916	GQ342758	
CBS 648.78	*Lichtheimia sphaerocystis* _c7				GQ342762

Anexo II: Abreviaturas

ADN = DNA	Acido desoxiribonucleico
AFST	*Antifungal Susceptibility Testing*
AMB	Anfotericina B
AND = ADF	Anidulafungina
ARN = RNA	Acido ribonucleico
ATCC	*American Type Culture Collection*
BenA	β tubulina
CAS = CPF	Caspofungina
CBS	*Fungal Biodiversity Centre*
CDC	*Centre for Disease Control and Prevention*
CECT	Colección Española de Cultivos Tipo
CIF = FIC	Concentración inhibitoria Fraccionaria (*Fractional Concentration Index*)
CLSI	*Clinical Laboratory Standards Institute*
CME = MEC	Concentración Mínima Efectiva (*Minimal Effective Concentration*)
CMI = MIC	Concentración Mínima Inhibitoria *(Minimal Inhibitory Concentration*)
COI	Citocromo C oxidasa mitocondrial subunidad I (*Mitochondrial cytochrome c oxidase subunit* I)
DDBJ	*DNA Databank of Japan*
EF-1α	Factor de elongación 1 α
EMBL	*European Molecular Biology Laboratory*
EMEA	*European Medicine Agency*
EUCAST	*European Committee on Antimicrobial Susceptibility Testing*
FDA	*Food and Drug Administration*
GCPSR	*Genealogical Concordance Phylogenetic Species Recognition*
IGS	Espaciador intergénico (*Intergenic Spacer Region)*
ITC	Itraconazol
ITS	Espaciador interno transcrito (*Internal transcribed spacer)*
LAmB	Anfotericina B liposomal *(Liposomal Amphotericin B*)
LSU	Subunidad grande ribosómica (*Ribosomal large subunit)*
MG = GM	Media Geométrica *(Geometric Mean*)
MICA= MCF	Micafungina
mtSSU	Subunidad pequeña mitocondrial *(mitochondrial ribosomal small subunit)*
NCBI	*National Centre for Biotechnology Information*
pb=bp	Pares de bases (*base pairs*)
PCR	Reacción en cadena de la polimerasa (*Polymerase chain reaction)*
PSC=POS	Posaconazol
RPBII	RNA polimerasa II segunda subunidad más grande (*Second largest subunit of the RNA polymerase II)*
rRNA	RNA ribosómico

RVZ= RCZ Ravuconazol

SSELO *Sequence Specific End Labelling*

TRB= TBF Terbinafina

VCZ= VRC Voriconazol